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(71) Applicant: THE REGENTS OF THE UNIVERSITY OF CALIFORNIA [US/US]; 2199 Addison Street, Berkeley, CA 94720 (US).

(72) Inventors: LONGO, Frank, M. ; 2175 Green Street No. 5, San Francisco, CA 94123 (US). MOBLEY, William, C. ; 294 Fernwood Drive, Moraga, CA 94556 (US). KAUER, James, Charles ; 605 Willow Glen Road, Kennett Square, PA 19348 (US).

(74) Agents: BENZ, William, H. et al.; Irell & Manella, 545 Middlefield Road, Suite 200, Menlo Park, CA 94025 (US).

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(57) Abstract

The present invention provides both agonist and antagonist nerve growth factor peptides. The NGF blocking peptides can be used to inhibit the expression of mRNA and their encoded proteins whose expression is stimulated by NGF, such as β-protein precursor and prior proteins, which proteins or their products are associated with neurodegenerative disorders, whereas the NGF agonist peptides can be used to treat neoplastic disorders such as neuroblastomas.

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NERVE GROWTH FACTOR PEPTIDES

Technical Field

This invention relates to neurotrophic factors and their involvement in neurodegenerative disorders. More particularly, this invention provides discrete peptides relating to nerve growth factor.

Background

Nerve growth factor (NGF) is a protein which has prominent effects on developing sensory and sympathetic neurons of the peripheral nervous system. NGF acts via specific cell surface receptors on responsive neurons to support neuronal survival, promote neurite outgrowth, and enhance neurochemical differentiation. NGF actions are accompanied by alterations in neuronal membranes (Connolly et al, (1981) J Cell Biol 90:176-180; Skaper and Varon (1980) Brain Res 197:379-389), in the state of phosphorylation of neuronal proteins (Yu et al, (1980) J Biol Chem 255:10481-10492; Halegoua and Patrick (1980) Cell 22:571-581), and in the abundance of certain mRNA's and proteins likely to play a role in neuronal differentiation and function (see, for example, Tiercy and Shooter (1986) J Cell Biol 103:2367-2378).

Forebrain cholinergic neurons also respond to NGF and may require NGF for trophic support. Indeed, the distribution and ontogenesis of NGF and its receptor in the central nervous system (CNS) suggest that NGF acts as a target-derived neurotrophic factor for basal forebrain

cholinergic neurons (Korsching, (1986) TINS, Nov/Dec, pp 570-573).

Deciphering the causation of human degenerative neurologic disorders has assumed increased importance with the realization that an increasing portion of the population may be affected. Trophic factors, such as NGF, are envisioned to have an important role in the development and maintenance of neurons (Crutcher (1986) CRC Crit Rev Clin Neurobiol 2:297-333), and it has been suggested that deficiencies in the supply or action of trophic factors may contribute to the loss of neurons in a number of neurodegenerative diseases (Appel (1981) Ann Neurol 10:499-505; Hefti and Weiner (1986) ibid 20:275-281).

As stated earlier, cholinergic neurons in the CNS also depend on NGF for survival. One major cholinergic pathway, from the basal forebrain to the hippocampus and neocortex (which responds to NGF), shows early, severe, relatively selective destruction in Alzheimer's disease. It is believed by some workers that NGF may aid in the survival and function of this neuronal population and thus provide a means for therapeutic treatment of Alzheimer's disease.

Amyloid plaques accumulate in the brain in various pathological states: Alzheimer's, Down's syndrome, normal aging, kuru, Creutzfeldt-Jacob disease, Gerstmann-Straussler syndrome, and other neurodegenerative disorders. The extent of amyloid accumulation in Alzheimer's brains correlates with the degree of mental impairment. It is possible that amyloid deposits cause such impairments. The mass of the accumulation itself can physically displace normal brain tissue (Mobley et al, (1983) Soc Neurosci 9:270). In addition, one of the amyloid proteins may promote neurite growth (Yanker et al (1988) Soc Neurosci 14:896; Schubert et al (1988) Science 241:223-226). In some of the above pathological states, massive tangles of neurites are associated with amyloid

plaques. Inappropriate neurite growth could alter the maintenance and detour the formation of normal neurite-target connections.

While there has been a proposal that the accumulation of amyloid could be due to excessive amounts of NGF, up until the present invention, there has been no reported demonstration of NGF's involvement in the promotion of amyloid synthesis in animal brain.

It is also known that NGF is capable of encouraging tumor metastasis (Glinsky et al, (1987) Annal New York Acad Sci 496:656-659).

The general concept of using some portion of a protein to block the binding of the entire protein to its intended receptor is known. For example, the peptide Gly-Arg-Gly-Asp-Ser inhibits the colonization of a murine melanoma cell line in lungs of mice into which the melanoma cells were injected (Humphries et al, (1986) Science 255:467-470). This is apparently a result of inhibiting the ability of these cells to bind to the fibronectin, an extracellular matrix which contains this pentapeptide sequence (Akiyama et al, (1985) J Biol Chem 260:10402-10405). Also, the peptide His-Glu-Pro-Pro was able to compete with IgE (of which it is a subsequence) for binding to F_CC receptors in human basophils; this tetrapeptide is thus considered a promising drug for the treatment of allergies (Prenner, (1987) Annal of Allergy 58:332-335).

A subunit of NGF has been proposed by Mercanti et al, (1977) Biochem Biophys Acta 494:412-419 to be an agonist of the full length molecule. However, Romani et al, (1987) Int J Peptide Protein Res 29:99-106; ibid. 107-117, after synthesizing this sequence (designated 10-25/75-88, according to the sequence correspondence in the disulfide linked dimer of identical 118 residue chains), analyzed the activity. The analysis showed that analog activity was not present in the synthetic fragment, nor

was this subunit capable of inhibiting binding of NGF, although tested at very high concentration relative to the NGF itself.

Thus, while some peptides of NGF have been synthesized, to date, no one has identified biologically active peptides that retain one or more of the biological activities associated with NGF, nor have any NGF antagonists been identified.

Disclosure of the Invention

The present invention provides a peptide having the ability to induce an NGF-associated biological response, wherein greater than 50% of the amino acid sequence of the peptide is at least 20% homologous with a receptor binding domain of NGF.

The present invention also provides NGF-related receptor-binding peptides having substantial homology to three discrete regions of native NGF. More particularly, one group of peptides comprises from 3 to 28 amino acid residues having substantial homology to residues 20-48 of native NGF (referred to at times herein as "Region C", with particular peptides within this region at times referred to as "C1", "C2", etc.), more preferably to residues 28-38 of NGF; a second group of peptides comprises from 3 to 34 amino acid residues having substantial homology to residues 50-84 of native NGF ("Region A"), more preferably to residues 66-75 of NGF; and a third group of peptides comprises from 3 to 25 amino acid residues having substantial homology to residues 87-112 of native NGF ("Region B"), more preferably to residues 98-106 of NGF.

Table 1, located herein in the Modes for Carrying Out the Invention section of this application, sets out the human and murine native sequences of the A, B, and C regions and lists the native sequences of the A1-A4, B1-B4 and C1-C13 subregions.

The peptides of this invention include the native regions and subregions as well as analogs of these sequences. These analogs include peptides which vary from the native material in any of the following ways:

1. Chemical modification of the amino and carboxyl groups present at the respective ends of the peptides.

2. Replacement of one or more of the amino acid residues in the native sequence with biologically compatible other amino acid resides.

3. Replacement of one or more of the amino acid residues in the native sequence with chemically modified biologically compatible other amino acid resides.

4. Deletion of one or more of the amino acid residues in the native sequence.

5. Repetition of one or preferably a sequence of several amino acid residues in the native sequence, with or without chemical modification to or replacement or deletion of one or more of the members of the sequence so as to give rise to "dimers", "trimers" and "polymers".

6. Cyclization, that is joining the ends, of a sequence of preferably a repeated sequence, such as a "dimer", etc.

The invention also provides as a preferred subgroup within the materials described above analogs of C2 NGF-related peptides exhibiting both agonist and antagonist properties in NGF bioassays. These analogs have the sequence:

$$R_1\text{-}AA_1\text{-}AA_2\text{-}AA_3\text{-}AA_4\text{-}AA_5\text{-}AA_6\text{-}AA_7\text{-}AA_8\text{-}AA_9\text{-}R_2$$

wherein:

AA_1 is a residue of an amino acid selected from the group Asp, d-Asp, Glu, d-Glu, Asn, d-Asn, Gln, d-Gln, and pyro-Glu, or can be deleted;

AA_2 is a residue of an amino acid selected from the group Ile, d-Ile, Val, d-Val, AdaA, AdaG, Leu, d-Leu, Met, and d-Met, or can be deleted;

AA_3 is a residue of an amino acid selected from the group Lys, d-Lys, Arg, d-Arg, Ile, d-Ile, Met, d-Met, homo-Arg, d-homo-Arg, Orn, and d-Orn, or can be deleted;

AA_4 is a residue of an amino acid selected from the group Gly, Ala, d-Ala, Pro, d-Pro, Aib, β-Ala, and Acp, or can be deleted;

AA_5 is a residue of an amino acid selected from the group Lys, d-Lys, Arg, homo-Arg, d-homo-Arg, Met, Ile, d-Arg, d-Met, d-Ile, Orn, and d-Orn, or can be deleted;

AA_6 is a residue of an amino acid selected from the group Glu, d-Glu, Gln, d-Gln, Asn, Asp, d-Asp, and d-Asn, or can be deleted;

AA_7 is a residue of an amino acid selected from the group Val, d-Val, Leu, d-Leu, Ile, d-Ile, Met, Chg, Cha, and d-Met, or can be deleted;

AA_8 is a residue of an amino acid selected from the group Thr, d-Thr, allo-Thr, Met, d-Met, Met(O) and d-Met(O), Ser, and d-Ser, or can be deleted;

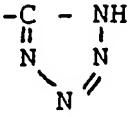
AA_9 either is a residue of an amino acid selected from the group Val, d-Val, Leu, d-Leu, Chg, and Valinol, or together with R_2 forms

$-NH-\underset{\displaystyle CH_2COOH}{\underset{|}{CH}}-R_4$ or $-NH-\underset{\displaystyle (CH_2)_2COOH}{\underset{|}{CH}}-R_4$, or can be deleted;

R_1 is attached to the amino group of AA_1 and selected from the group of hydrogen, lower alkyl, lower alkyl carbonyl, lower alkenyl, lower alkynyl, formyl, aryl, aroyl, aryloxy-carbonyl, aralkyloxy-carbonyl, lower alkyloxycarbonyl, benzoyl, 1- or 2-thenoyl, nicotinoyl, dihydronicotinoyl, N-alkyldihydronicotinoyl, isonicotinoyl, N-alkyldihydroisonicotinoyl, tetrahydronicotinoyl, and N-alkyltetrahydronicotinoyl;

R_2 is attached to the carbonyl group carbon of AA_9 and selected from the group of OH, NH_2, OR_3, OR_3OH, and NHR_3 with NH_2 and NHR_3 being preferred;

R_3 is selected from the group of lower alkyl and aryl groups; and

R_4 is selected from the group of PO_3H_2, $B(OH)_2$, CH_2OH, SO_3H, and  .

Preferably at least five, and even more preferably at least eight, of AA_1 through AA_9 are selected from the native NGF C2 sequence in which AA_1 is Asp, AA_2 is Ile, AA_3 is Lys, AA_4 is Gly, AA_5 is Lys, AA_6 is Glu, AA_7 is Val, AA_8 is Thr, and AA_9 is Val. Other preferred configurations in the C2 region employ the native sequence with R_2 as NH_2 or the native sequence with the AA_8 Thr deleted and R_2 as NH_2.

Yet other materials of the invention are analogs of the C5 NGF-related peptide. These display strong NGF antagonist properties and have the sequence:

R_1-AA_1-AA_2-AA_3-AA_4-R_2 wherein:

AA_1 is a residue of an amino acid selected from the group Lys, d-Lys, Arg, d-Arg, Pro, d-Pro, Nle, homo-Arg, d-homo-Arg, Orn and d-Orn or can be deleted.

AA_2 is a residue of an amino acid selected from the group consisting of: Val, d-Val, Gly, Pro, d-Pro, Ala, d-Ala, Aib, Leu, d-Leu, Ile, and d-Ile or can be deleted;

AA_3 is a residue of an amino acid selected from the group Lys, d-Lys, Arg, d-Arg, Pro, d-Pro, and Nle or can be deleted;

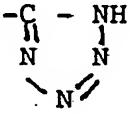
AA_4 is a residue of an amino acid selected from the group consisting of: Glu, d-Glu, Gln, d-Gln, Asp, d-Asp, Asn, d-Asn, Phe, and d-Phe, or can be deleted, or together with R_2 forms $-NH-\underset{\underset{CH_2COOH}{|}}{CH}-R_4$ or $-NH-\underset{\underset{(CH_2)_2COOH}{|}}{CH}-R_4$;

R_1 is attached to the amino group of AA_1 and selected from the group of hydrogen, lower alkyl, lower alkyl carbonyl, lower alkenyl, lower alkynyl, formyl,

aryl, aroyl, aryloxy-carbonyl, aralkyloxy-carbonyl, lower alkyloxycarbonyl, benzoyl, 1- or 2-thenoyl, nicotinoyl, dihydronicotinoyl, N-alkyldihydronicotinoyl, isonicotinoyl, N-alkyldihydroisonicotinoyl, tetrahydronicotinoyl, and N-alkyltetrahydronicotinoyl;

R_2 is attached to the carbonyl group carbon of AA_4 and selected from the group of OH, NH_2, OR_3, OR_3OH, and NHR_3 with NH_2 and NHR_3 being preferred;

R_3 is selected from the group of lower alkyl and aryl groups; and

R_4 is selected from the group of PO_3H_2, $B(OH)_2$, CH_2OH, SO_3H, and  .

Preferably at least two, and more preferably three, of AA_1, AA_2, AA_3 and AA_4 are selected from the native C5 NGF sequence in which AA_1 is Lys, AA_2 is Gly, AA_3 is Lys and AA_4 is Glu, preferably with R_2 as NH_2 or NHR_3. Also it is preferred that the C5 materials contain at least 3 and at most 5 amino acid residues.

When any peptide of the invention includes a glutamic or aspartic acid residue, its free OH group can be replaced by any of the groups listed above for R_2.

The invention also provides a method of inhibiting NGF-mediated biochemical or biophysical cellular effects which comprises applying an NGF antagonist peptide to NGF responsive cells.

Another aspect of the invention is a method of inhibiting the expression of mRNA or their encoded proteins by cells, the expression of which is stimulated by NGF, wherein the method comprises applying an NGF antagonist peptide of the invention to the cells.

Yet another aspect of the invention is a method for treatment of disorders involving NGF-responsive cells, which method comprises administering an effective amount

of one or more of the present peptides to an individual suffering from such a disorder.

Pharmaceutical compositions composed of the peptides are also disclosed herein.

Brief Description of the Drawings

Figures 1A and 1B are graphs which depict the results of NGF antagonist inhibition assays using increasing concentrations of the C1, C2, and C5 peptide and a fixed concentration of NGF (1A), or increasing concentrations of NGF with a fixed concentration of C5 (1B). The NGF bioeffect being referred to is the promotion of neurite outgrowth.

Figure 2 demonstrates agonist activity of C2 peptide.

Figure 3 is a dose/response curve for the effect of CNTF on day 10 dorsal root ganglion neurons. C5 had no effect on CNTF activity.

Figure 4 is a comparison of the concentration ranges through which C5 inhibits the NGF bioeffect (i.e. neurite outgrowth) without altering NGF binding.

Figures 5A and 5B illustrate the response of ChAT activity and of PrP mRNA levels to NGF treatment.

Figure 6 demonstrates the response of β-protein precursor mRNA to NGF treatment.

Modes for Carrying Out the Invention

A. Definitions

For purposes of the present invention the following terms are defined below.

"Agonist" refers to an NGF peptide capable of promoting at least one of the biological responses normally associated with NGF. For example, an NGF agonist may promote neurite growth but fail to enhance neurochemical differentiation in NGF responsive neurons.

"Antagonist" refers to an NGF peptide that opposes at least one of the effects of NGF. In the presence of an antagonist, NGF has reduced ability to mediate biological responses normally associated with NGF.

An NGF receptor binding domain is any region of the NGF molecule which interacts directly or indirectly with the NGF receptor. Such interaction is intended to include any covalent or non-covalent interactions. The putative receptor binding domains for NGF, as numbered to correspond to the murine β-NGF sequence, include amino acid residues 20-48 (Region C), 50-84 (Region A) and 87-112 (Region B).

"Substantial homology" as used herein refers to substantial correspondence between the identity and sequence of amino acid residues of the native, murine NGF protein and the synthetic peptides. More particularly, at least half of the residues in the synthetic peptides correspond to the residues present in the native molecule.

Murine NGF may be readily isolated from the submaxillary gland of male mice. This murine NGF is expressed as a 7S complex, containing the NGF-α, β and δ subunits. Alternatively, the NGF may be produced by recombinant DNA techniques as disclosed in European Patent Publication 121,338, published 10 October 1984, the disclosure of which is incorporated herein by reference. β-NGF is a dimer of two identical 118 amino acid chains, and is apparently solely responsible for the observed biological activity of NGF. As used herein, "NGF" refers to β-NGF.

The primary sequence for NGF molecules from five different species have been determined (Meier et al, (1986) EMBO J 5(7):1489-1493). Studies on the biological relatedness of NGF's purified from different species strongly support the hypothesis that the site (or sites) of interaction with their receptors has remained structurally constant. For purposes of the present invention, the

NGF sequence can be derived from any mammalian source, including, for example, murine or human origin, since the homology between the receptor binding domains among mammalian species is extremely high. Table 1 shows the comparison for Regions A, B and C between murine and human NGF.

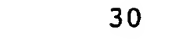
The term "effective amount" refers to the amount of NGF peptide needed to produce a desired manifestation or blocking of NGF activity. The precise amount will vary with the particular NGF peptide employed, the age and condition of the subject to be treated, and the nature and severity of the condition. However, the effective amount may be determined by one of ordinary skill in the art with only routine experimentation, following the methodology described herein.

As used herein, the terms "NGF-mediated activity" or "NGF-associated activity" refers to cellular events triggered by NGF. The nature of these activities may be biochemical or biophysical. The following list is provided, without limitation, which discloses some of the known activities associated with NGF: ion flux, phospholipid metabolism, activation of cyclic AMP-dependent protein kinase, activation of cyclic AMP-independent protein kinase and other protein kinases, protein phosphorylation, activation of oncogenic proteins, activation of RNA transcription, stabilization of mRNA species, and enhancement of protein synthesis.

The terms "disorder" or "neurodegenerative disorder" as used herein refer to a disease state in a mammal which can include degenerative growth and development disorders, chromosomal abnormalities, viral infections and disorders of the nervous system or neoplastic conditions which can respond to treatment with the NGF-derived peptides. Thus, diseases characterized by the loss of function and/or degeneration of neurons and nerves are within the scope of the invention. In addition, any

disease that can respond to treatment of NGF-responsive or NGF-synthesizing cells with the present peptides is within the scope of the invention. Exemplary cell types include without limitation, cholinergic neurons, sensory and sympathetic neurons, adrenal medullary cells, Schwann cells, fibroblasts, smooth muscle cells, astroglial cells, macrophages, epithelial cells, and melanoma cells. Exemplary disorders include without limitation, Alzheimer's disease, Down's syndrome, Creutzfeldt-Jacob disease, kuru, Gerstman-Straussler syndrome, scrapie, transmissible mink encephalopathy, Huntington's disease, Riley-Day familial dysautonomia, multiple system atrophy, and the like. Also considered within this definition is the treatment of injury to the nervous system. Additionally, neural tumors, such as neuroblastoma, involving either neurons or their supporting elements, may be responsive to treatment with NGF and NGF agonists. NGF antagonists would find utility in treating pathological events which may be enhanced by NGF, such as the treatment of tumors or tumor metastasis (Ginsky, supra).

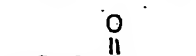
The amino terminal substituent (R_1) of the NGF peptides includes: hydrogen, lower alkyls having from 1 to 4 carbons such as methyl, ethyl, propyl and butyl and their amide-yielding lower alkyl carbonyl forms such as formyl and acetyl; lower alkenyls, such as ethenyl, and prop-1-enyl and prop-2-enyl; lower alkynyls such as ethynyl and the propynyls; an aryl group such as phenyl or naphthyl; an aroyl group such as benzoyl or naphthoyl; an aryloxy-carbonyl such as



; an aralkyloxy-carbonyl such as

; and a lower alkyloxycarbonyl such as

or



. Hydrogen is a preferred R_1 substituent.

The carboxy terminal substituent (R_2) of the NGF peptides includes: OH; NH_2; and OR_3, such that the carboxy terminus is a lower alkyl or aryl ester, wherein R_3 is a lower alkyl having from 1 to 4 carbons or an aryl group of 6 to 10 carbons; OR_3OH such that the terminus is a hydroxy-substituted ester with R_3 defined as above; NH-R_3, wherein R_3 is defined as above, and PO_3H_2, $B(OH)_2$, CH_2OH, SO_3H and 5-tetrazole which replaces the COOH of the carboxyl-terminal amino acid.

B. NGF Peptides

The nomenclature used to define the peptides is that specified by Schroder & Lubke, "The Peptides", Academic Press (1965), wherein in accordance with conventional representation, the amino group at the N-terminus appears to the left and the carboxyl group at the C-terminus to the right. By natural amino acid is meant one of common, naturally occurring amino acids found in proteins comprising Ala (A), Arg (R), Asn (N), Asp (D), Cys (C), Gln (Q), Glu (E), Gly (G), His (H), Ile (I), Leu (L), Lys (K), Met (M), Phe (F), Pro (P), Ser (S), Thr (T), Trp (W), Tyr (Y) and Val (V). By Nle is meant norleucine, by Aib is meant aminoisobutyric acid, by AdaA is meant α-adamantylalanine, by AdaG is meant β-adamantylglycine, by homo-Arg is meant l-homoarginine, by d-homo-Arg is meant d-homoarginine, by Acp is meant epsilon-amino caproic acid, by Chg is meant l-α-cyclohexylglycine, and by allo-Thr is meant l-allothreonine. Additionally, by Cha is meant β-cyclohexyl-alanine, by Me is meant methyl (CH_3), by Orn is meant ornithine, by pyro-Glu is meant the pyroglutamyl group, by Met(O) and d-Met(O) are meant the sulfoxides derived from l- and d-methionine, respectively, and by β-Ala is meant β-alanine.

The symbolism and abbreviations used are otherwise those recommended by the "IUPAC-IUB Joint Commission on Biochemical Nomenclature, Nomenclature and Symbolism

for Amino Acids and Peptides, Recommendations 1983." As is conventional, these same symbols will be used to define the corresponding residues of the acids when they are linked into a peptide chain. Where the amino acid residue has isomeric forms, it is the L-form of the amino acid that is represented unless otherwise expressly indicated.

Studies on the effects of exogenous NGF have provided important clues regarding the role of NGF in the normal growth and development of target neurons. The data which most clearly indicates a physiological role for endogenous NGF comes from studies of the effect of NGF antibodies on the growth and development of responsive neuronal populations. A significant limitation in the performance of antibody experiments, and their interpretation, lies in the degree to which antibodies have access to NGF. This limitation is especially noteworthy for studies of NGF actions in the central nervous system.

Another method for studying the role of trophic factors such as NGF, involves inhibiting the action of polypeptides by blocking binding of the polypeptide to its specific receptor. The primary requirement is to identify regions of the polypeptide likely to interact with these receptors. Though there is a great deal of data regarding structural and functional characteristics of the interaction of NGF with its receptor (see Yankner and Shooter (1982) Ann Rev Biochem 51:845-868), the regions of NGF involved in receptor binding are as yet unknown. If one predicts that NGF interacts with its receptor via hydrophilic domains, and that these domains are highly conserved through evolution, one can make informed guesses as to which regions may mediate binding. However, there are no clear criteria for quantitatively predicting the degree of agonist or antagonist activity of a given peptide.

From the known amino acid sequence of NGF, three regions were investigated as possible receptor binding domains. Region A (residues 50-84), Region B (residues

87-112) and Region C (residues 20-48) were chosen because of their relatively high degree of hydrophilicity, cross-species conservation, or the probability of containing a β-turn. The specific peptides listed in Table 1 below, each synthesized with their carboxy terminus in the amide form, were tested for their ability to block NGF activity or, in the case of agonists, to support neuronal survival as described in the examples which follow. The sequences provided for each of the three regions are provided; the human sequence is illustrated beneath the murine sequence.

TABLE 1

Native Amino Acid Sequences For
A, B and C Regions and Subregions

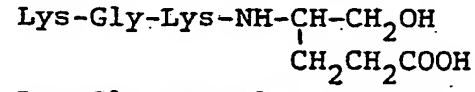
Region A:	Region B:	Region C:
Murine:	Murine:	Murine:
SGCRGIDSKH	AWRFIRIDT	ATDIKGKEVTV
Human:	Human:	Human:
SGCRGIDSKH	AWRFIRIDT	ATDIKGKEVMV
A1 SGCRGIDSKH	B1 AWRFIRIDT	C1 ATDIKGKEVTV
A2 RGIDSKH	B2 RIDT	C2 DIKGKEVTV
A3 RGIDS	B3 RFIRIDT	C3 KGKEVTV
A4 RDISDK	B4 RFIRID	C4 DIKGKE
		C5 KGKE
		C6 DIKG
		C7 KGK
		C8 IKG
		C9 IKGK
		C10 ATDIKG
		C11 GKEV
		C12 GKE
		C13 KG

Other representative analogs falling within the scope of this invention include the following C5 and C2 analogs:

C5 ANALOGS

Glu-4 Modifications

Lys-Gly-Lys-Glutamic Acid

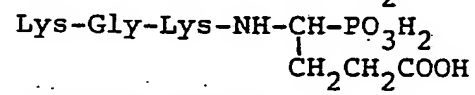


Lys-Gly-Lys-Glu-α-NH-CH_3

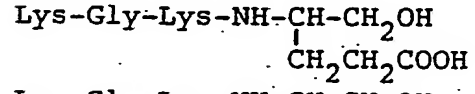
Lys-Gly-Lys-Gln-OH

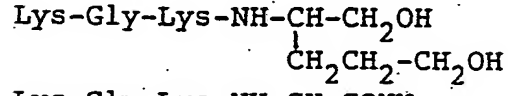
Lys-Gly-Lys-Gln-NH_2

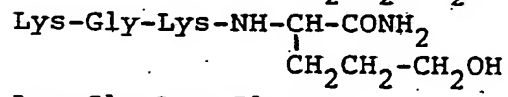
Lys-Gly-Lys-d-Glu-NH_2



Lys-Gly-Lys-Gln-NH_2







Lys-Gly-Lys-Glu-NH_2

Lys-Gly-Lys-Phe-NH_2

Lys-3 Modifications

Lys-Gly-Arg-Glu-NH_2
Lys-Gly-Arg-Glu-NH-CH_3
Lys-Gly-Arg-Gln-OH
Lys-Gly-Nle-Glu-NH_2
Lys-Gly-Pro-Glu-NH_2
Lys-Gly-Pro-NH-CH(CH_2CH_2COOH)-PO_3H_2

Gly-2 Modifications

Lys-Pro-Lys-Asp-NH-CH_3
Lys-Ala-Lys-Glu-NH-CH_3
Lys-Val-Lys-Glu-NH_2
Lys-Pro-Lys-Glu-NH-CH_3
Lys-d-Ala-Lys-Glu-NH_2
Lys-Ala-Lys-Glu-NH_2
Lys-d-Ala-Lys-Glu-NH_2
Lys-Aib-Lys-Glu-NH_2
Lys-Leu-Lys-Glu-NH_2

Lys-1 Modifications

Orn-Gly-Lys-Glu-NH_2
d-Orn-Gly-Lys-Glu-NH_2
d-Lys-Gly-Lys-Glu-NH_2
d-Lys-d-Ala-Lys-Glu-NH_2
d-Lys-Gly-Lys-Glu-NH-CH_3
Arg-Gly-Lys-Glu-NH_2
d-Arg-Gly-Lys-Glu-NH_2
Arg-Gly-Lys-Glu-NH-CH_3
Arg-Gly-Arg-Glu-NH_2
d-Lys-Gly-Arg-Asp-NH_2
homo-Arg-Gly-Lys-Glu-NH_2
d-homo-Arg-Gly-Lys-Glu-NH_2

Dimers

Lys-Gly-Lys-Glu-Lys-Gly-Lys-Glu-NH_2

Lys-Gly-Lys-Glu-β-Ala-Lys-Gly-Lys-Glu-NH_2

Lys-Gly-Lys-Gln-β-Ala-Lys-Gly-Lys-Gln-NH_2

Polymeric Peptides Wherein n is an Integer Between 2 and 40

$(Lys\text{-}Gly\text{-}Lys\text{-}Glu)_n\text{-}R_2$

$(Lys\text{-}Gly\text{-}Lys\text{-}Glu(NH_2))_n\text{-}R_2$

$(Lys\text{-}Gly\text{-}Lys\text{-}Glu\text{-}Gly)_n\text{-}R_2$

$(Lys\text{-}Gly\text{-}Lys\text{-}Glu\text{-}\beta\text{-}Ala)_n\text{-}R_2$

$(Lys\text{-}Gly\text{-}Lys\text{-}Glu\text{-}Pro)_n\text{-}R_2$

Cyclic Analogs

Cyclo(-Lys-Gly-Lys-Glu-Lys-Gly-Lys-Glu-)

Cyclo(-Lys-Gly-Lys-Glu-Pro-Lys-Gly-Lys-Glu-Pro-)

Cyclo(-Lys-Gly-Lys-Glu-β-Ala-Lys-Gly-Lys-Glu-β-Ala-)

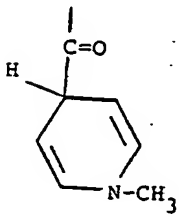
Selective acylation of side chain NH_2 group of Lys-1 or 3

Lys(Ac)-Gly-Lys-Glu-NH_2

Lys-Gly-Lys(Ac)-Glu-NH_2

Lys-Gly-Lys(Bz)-Glu-NH_2

Lys-Gly-Lys-Glu-NH_2



Lys-Gly-Lys-Glu-Acp-analogs:

Lys-Gly-Lys-Glu-Acp-OH
Lys-Gly-Lys-Glu-Acp-NH_2
Lys-Gly-Lys-Glu-$NH(CH_2)_6$-OH
Lys-Ala-Lys-Glu-Acp-OH
Lys-Pro-Lys-Glu-Acp-OH
Lys-Ala-Lys-Glu-Acp-NH_2
Lys-Pro-Lys-Glu-Acp-NH_2
Lys-Pro-Lys-Glu-$NH(CH_2)_6$-OH

C2 ANALOGS

Asp-1 Modifications

d-Asp-Ile-Lys-Gly-Lys-Glu-Val-Thr-Val-R_2*
d-Asp-Ile-Lys-Gly-Lys-Glu-Val-Thr-Val-OH
d-Asp-Ile-Lys-Gly-Lys-Glu-Val-Thr-Val-OCH_3
d-Asp-Ile-Lys-Gly-Lys-Glu-Val-Thr-Val-NH_2
d-Asp-Ile-Lys-Gly-Lys-Glu-Val-Thr-Val-NH-CH_3
d-Asp-Ile-Lys-Gly-Lys-Glu-Val-Thr-Val-PO_3H_2
Glu-Ile-Lys-Gly-Lys-Glu-Val-Thr-Val-R_2
Asn-Ile-Lys-Gly-Lys-Glu-Val-Thr-Val-R_2
Gln-Ile-Lys-Gly-Lys-Glu-Val-Thr-Val-R_2
d-Glu-Ile-Lys-Gly-Lys-Glu-Val-Thr-Val-R_2
d-Gln-Ile-Lys-Gly-Lys-Glu-Val-Thr-Val-R_2

* R_2 as defined above.

Ile-2 Modifications

Asp-Leu-Lys-Gly-Lys-Glu-Val-Thr-Val-R_2
Asp-Val-Lys-Gly-Lys-Glu-Val-Thr-Val-R_2
Asp-AdaA-Lys-Gly-Lys-Glu-Val-Thr-Val-R_2
Asp-AdaG-Lys-Gly-Lys-Glu-Val-Thr-Val-R_2
Asp-d-Ile-Lys-Gly-Lys-Glu-Val-Thr-Val-R_2
Asp-d-Leu-Lys-Gly-Lys-Glu-Val-Thr-Val-R_2
Asp-d-Val-Lys-Gly-Lys-Glu-Val-Thr-Val-R_2
Asp-Cha-Lys-Gly-Lys-Glu-Val-Thr-Val-R_2

Lys-3 Modifications

Asp-Ile-d-Lys-Gly-Lys-Glu-Val-Thr-Val-R_2
Asp-Ile-Arg-Gly-Lys-Glu-Val-Thr-Val-R_2
Asp-Ile-homo-Arg-Gly-Lys-Glu-Val-Thr-Val-R_2
Asp-Ile-d-Arg-Gly-Lys-Glu-Val-Thr-Val-R_2
Asp-Ile-Ile-Gly-Lys-Glu-Val-Thr-Val-R_2
Asp-Ile-Met-Gly-Lys-Glu-Val-Thr-Val-R_2
Asp-Ile-d-Orn-Gly-Lys-Glu-Val-Thr-Val-R_2
d-Gln-Leu-d-Lys-Gly-Lys-Glu-Val-Thr-Val-R_2
d-Asp-Leu-d-Lys-Gly-Lys-Glu-Val-Thr-Val-R_2

Gly-4 Modifications

Asp-Ile-Lys-Ala-Lys-Glu-Val-Thr-Val-R_2
Asp-Ile-Lys-Aib-Lys-Glu-Val-Thr-Val-R_2
Asp-Ile-Lys-Ala-Lys-Glu-Val-Thr-Val-R_2
Asp-Ile-Lys-β-Ala-Lys-Glu-Val-Thr-Val-R_2
Asp-Ile-Lys-Acp-Lys-Glu-Val-Thr-Val-R_2
d-Asp-Ile-Lys-d-Ala-Lys-Glu-Val-Thr-Val-R_2
Asp-Ile-Lys-d-Pro-Lys-Glu-Val-Thr-Val-R_2

Lys-5 Modifications

Asp-Ile-Lys-Gly-d-Lys-Glu-Val-Thr-Val-R_2
Asp-Ile-Lys-Gly-Arg-Glu-Val-Thr-Val-R_2
Asp-Ile-Lys-Gly-l-Arg-Glu-Val-Thr-Val-R_2
Asp-Ile-Lys-Gly-homo-Arg-Glu-Val-Thr-Val-R_2
Asp-Ile-Lys-Gly-Met-Glu-Val-Thr-Val-R_2
Asp-Ile-Lys-Gly-Ile-Glu-Val-Thr-Val-R_2
Asp-Ile-Lys-Pro-d-Arg-Glu-Val-Thr-Val-R_2
Asp-Ile-Lys-Pro-d-Orn-Glu-Val-Thr-Val-R_2

Glu-6 Modifications

Asp-Ile-Lys-Gly-Lys-d-Glu-Val-Thr-Val-R_2
Asp-Ile-Lys-Gly-Lys-Asp-Val-Thr-Val-R_2
Asp-Ile-Lys-Gly-Lys-d-Asp-Val-Thr-Val-R_2
Asp-Ile-Lys-Gly-Lys-Gln-Val-Thr-Val-R_2
Asp-Ile-Lys-Gly-Lys-Asn-Val-Thr-Val-R_2
Asp-Ile-Lys-Pro-Lys-d-Glu-Val-Thr-Val-R_2

Val-7 Modifications

Asp-Ile-Lys-Gly-Lys-Glu-Leu-Thr-Val-R_2
Asp-Ile-Lys-Gly-Lys-Glu-Ile-Thr-Val-R_2
Asp-Ile-Lys-Gly-Lys-Glu-Met-Thr-Val-R_2
Asp-Ile-Lys-Gly-Lys-Glu-l-Val-Thr-Val-R_2
Asp-Ile-Lys-Gly-Lys-Glu-Chg-Thr-Val-R_2
Asp-Ile-Lys-d-Pro-Lys-Glu-Leu-Thr-Val-R_2

Thr-8 Modifications

Asp-Ile-Lys-Gly-Lys-Glu-Val-Ser-Val-R_2
Asp-Ile-Lys-Gly-Lys-Glu-Val-d-Thr-Val-R_2
Asp-Ile-Lys-Gly-Lys-Glu-Val-allo-Thr-Val-R_2
Asp-Ile-Lys-l-Pro-Lys-Glu-Val-Ser-Val-R_2
Asp-Ile-Lys-l-Pro-Lys-Glu-Val-Leu-Val-R_2
Asp-Ile-Lys-Gly-Lys-Glu-Val-Met-Val-R_2
Asp-Ile-Lys-Gly-Lys-Glu-Val-Val-R_2

Val-9 Modifications

Asp-Ile-Lys-Gly-Lys-Glu-Val-Met-Leu-R_2
Asp-Ile-Lys-Gly-Lys-Glu-Val-Met-Met-R_2
Asp-Ile-Lys-Gly-Lys-Glu-Val-Met-Chg-R_2
Asp-Ile-Lys-Gly-Lys-Glu-Val-Met-Valinol

Dimers

(Asn-Ile-Lys-Gly-Lys-Gln-Val-Thr-Val)$_2$-NH_2
(Asp-Ile-Lys-Gly-Lys-Glu-Val-Thr-Val)$_2$-OH
(Asp-Ile-Lys-Gly-Lys-Glu-Val-Thr-Val)$_2$(Gly)-R_2
(Asp-Ile-Lys-Gly-Lys-Glu-Val-Thr-Val)$_2$(Pro)-R_2
(Asp-Ile-Lys-Gly-Lys-Glu-Val-Thr-Val)$_2$β-Ala-R_2

Cyclic Analogs

Cyclo(-Asp-Ile-Lys-Gly-Lys-Glu-Val-Thr-Val-)
Cyclo(-Asp-Ile-Lys-Gly-Lys-Gln-Val-Thr-Val-)
Cyclo(-Asp-Ile-Lys-Gly-Lys-Glu-Val-Thr-Val-Gly-)
Cyclo(-Asp-Ile-Lys-Gly-Lys-Glu-Val-Thr-Val-β-Ala-)

Polymeric Peptides Wherein n is an Integer Between 3 and 20

(Asp-Ile-Lys-Gly-Lys-Glu-Val-Thr-Val)$_n$-R_2

Selective acylation of Lys side chain NH_2 group

The amino-terminal amino group and/or the lysine or threonine side chains may optionally be acylated by formyl, acetyl, propionyl and similar lower alkylacyl residues or by aryl or heterocyclic acyl residues such as benzoyl, thenoyl, nicotinoyl, isonicotinoyl, n-alkylnicotinoyl and their dihydro and tetrahydro derivatives. Such modifications will enhance the blood-brain-barrier permeability of the therapeutic agent. (C.R. Creveling et al, (1969) Experientia 25:26-27 and N. Bodor et al. (1981) Science 214:1370-1372.

It should be noted that the above C5 and C2 analogs are representative only and additional modifications can be made to other positions in accordance with the present invention.

C. Preparation Methods

The peptides of the present invention may be synthesized by any techniques that are known to those skilled in the peptide art. An excellent summary of the many techniques so available may be found in J.M. Stewart and J.D. Young, Solid Phase Peptide Synthesis, 2nd Ed., Pierce Chemical Co., Rockford, IL (1984) and G. Barany and R.B. Merrifield, The Peptides: Analysis, Synthesis, Biology, Editors E. Gross and J. Meienhofer, Vol. 2, Academic Press, New York (1980), pp. 3-254, for solid phase peptide synthesis; and M. Bodansky, Principles of Peptide Synthesis, Springer-Verlag, Berlin (1984) and E. Gross and J. Meienhofer, Eds., The Peptides: Analysis, Synthesis, Biology, supra, Vol. 1, for classical solution synthesis.

In general, these methods comprise the sequential addition of one or more amino acids or suitably protected amino acids to a growing peptide chain. Normally, either the amino or carboxyl group of the first amino acid is protected by a suitable protecting group. The protected or derivatized amino acid can then be either

attached to an inert solid support or utilized in solution by adding the next amino acid in the sequence having the complementary (amino or carboxyl) group suitably protected, under conditions suitable for forming the amide linkage. The protecting group is then removed from this newly added amino acid residue and the next amino acid (suitably protected) is then added, and so forth. After all the desired amino acids have been linked in the proper sequence, any remaining protecting groups (and any solid support) are removed sequentially or concurrently, to afford the final polypeptide. By simple modification of this general procedure, it is possible to add more than one amino acid at a time to a growing chain, for example, by coupling (under conditions which do not racemize chiral centers) a protected tripeptide with a properly protected dipeptide to form, after deprotection, a pentapeptide.

Typical protecting groups include t-butyloxycarbonyl (Boc), 9-fluorenylmethoxycarbonyl (Fmoc) benzyloxycarbonyl (Cbz); p-toluenesulfonyl (Ts); 2,4-dinitrophenyl; benzyl (Bzl); biphenylisopropyloxycarboxy-carbonyl, t-amyloxycarbonyl, isobornyloxycarbonyl, o-bromobenzyloxycarbonyl, cyclohexyl, isopropyl, acetyl, o-nitrophenylsulfonyl and the like. Of these, Boc is preferred.

Typical solid supports are generally classified as cross-linked polymeric supports. These can include divinylbenzene cross-linked styrene-based polymers, for example, divinylbenzene-hydroxymethylstyrene copolymers, divinylbenzene-chloromethylstyrene copolymers and divinylbenzene-benzhydrylaminopolystyrene copolymers. The last of these materials, as illustrated herein using p-methyl-benzhydrylamine resin, offers the advantage of directly introducing the terminal amide function into the peptide chain, which function is retained by the chain when the chain is cleaved from the support.

In the preferred practice of the invention, the peptides are prepared by conventional solid phase chemical synthesis on, for example, an Applied Biosystems, Inc. (ABI) 430A peptide synthesizer using a resin which permits the synthesis of the amide peptide form and t-Boc (tert-butyloxycarbonyl) derivatives (Peninsula Laboratories, Inc.) with standard solvents and reagents. Peptide composition is confirmed by quantitative amino acid analysis and the specific sequence of each peptide may be determined by sequence analysis.

Alternatively, peptides containing only natural amino acids can be produced by recombinant DNA techniques by synthesizing DNA encoding the desired peptide, along with an ATG initiation codon. Next the DNA is cloned into an appropriate expression vector, such as pBR322-type vectors, using conventional methods to amplify the DNA. Once the sequence has been suitably amplified, it may be transferred to an appropriate expression vector.

Selection of the particular expression vector depends upon the particular host used. For example, bacterial plasmids are used advantageously with E. coli cells. The expression vector preferably includes at least one regulatory element, which enables one to induce and/or suppress the expression of the introduced sequence. Thus, one may grow the host culture to a desired density prior to expressing the desired peptide and harvesting the product. If a fusion protein was produced, cleavage of the desired peptide from the fused protein is also performed.

The peptides may also be produced by enzymatic or chemical cleavage of purified NGF or a polypeptide having the desired sequence. Such procedures are conventional and well known in the art.

The original source for the derivation of the present peptides is NGF. However, a search through the library of peptide sequences (400,000 amino acid residues)

collected by the National Biomedical Research Foundation (George Washington University Medical Center, Washington, D.C.) demonstrated 22 peptides which contain KGKE (peptide C5). Of the 22 peptides found, 12 occur in eukaryotic cells as shown in Table 2 below. Thus, while the NGF-derived peptides have certain agonist or antagonist properties for NGF, they may also find utility as agonists or antagonists to one or more of the peptides described below.

TABLE 2

Peptides Containing KGKE

Apolipoprotein B-100 precursor - Human
Carbonic anhydrase III - Human, horse
Hypothetical polymerase - Fruit fly transposable element 17.6
Platelet basic protein - Human
Heat shock protein 26 - Fruit fly
5-aminolevulinate synthase precursor - Chicken
Gene PET494 protein - Yeast
Adenylate cyclase - Yeast
DNA-directed RNA polymerase II - Yeast, fruit fly
405 ribosomal protein S14 - Chinese hamster
Connective-tissue activating peptide III - Human
pol-like polyprotein - Fruit fly

After synthesis, the peptides of the present invention may then be assayed to determine their activity in the NGF bioassay using dissociated embryonic chick dorsal root ganglia neurons according to published procedures, such as, for example, Riopelle et al, (1981) J Neurobiol 12:175-186 or Varon et al (1981) Dev Brain Res 1:73-87. Any peptide of interest may be evaluated in this

assay to determine whether the peptide possesses antagonist or agonist activity with respect to NGF. If the peptide of interest is capable of inhibiting neuronal attachment, survival or neurite outgrowth in the presence of NGF, the peptide is defined as an antagonist. Similarly, if the peptide is capable of supporting neuronal attachment, survival or neurite outgrowth in the absence of NGF, it is defined as an agonist.

The versatility of this assay provides a convenient tool to assess whether a specific peptide of interest falls within the scope of the present invention. It should be understood that while particular species may or may not have utility as an agonist or antagonist, all are useful in the development of organic agonists and antagonists since lack of activity is important to understanding and defining the NGF binding site. Thus, additional peptides may be generated from other regions within NGF, including peptides containing amino acid substitutions or deletions, as well as other chemical modifications, which may lead to peptides with greater inhibitory activity or perhaps a more significant agonist effect as measured in the NGF bioassay. Other variations and modifications will be apparent to those of ordinary skill in the art, and are to be considered within the scope of the instant invention.

D. General Methods of Use

The peptides described herein can be used to treat a number of neurodegenerative or neoplastic disorders. Alzheimer's disease is a neurodegenerative disorder. One of the hallmarks of Alzheimer's disease is the presence of numerous neuritic plaques. These plaques consist of degenerating axons and neurites surrounding an amyloid plaque core composed of 5- to 10-nm proteinaceous filaments. Similar filaments are also found outside of plaques as cerebrovascular amyloid. The brains of aged

individuals with Down's syndrome also have both types of amyloid deposits. The β-protein is the principal protein component found in both cerebrovascular and neuritic plaque amyloid. A common mechanism may thus underlie the formation of amyloid in these diseases.

As demonstrated herein, NGF is shown to promote the expression of the genes for both the β-protein and the prion protein. An isoform of the latter protein is the infectious agent causing scrapie and is chemically related to the infectious agent(s) causing Creutzfeldt-Jacob disease, kuru, and to the host gene product causing Gerstmann-Straussler syndrome. Individuals suffering from these disorders or having a predisposition to these disorders may be treated with the NGF antagonist peptides of the invention to regulate the expression of these genes and their proteins, and therefore reduce the accumulation of the β-protein in Alzheimer's disease and Down's syndrome or the prior protein in CJD, kuru and GSS.

The agonist peptides of the present invention may also be used to treat neurodegenerative disorders caused by a lack of NGF, such as, for example, Huntington's disease. In this disease, cholinergic neurons in the caudate-putamen degenerate. As NGF has been shown to support the differentiation of cholinergic neurons, agonist peptides may be administered to enhance the activity of the cholinergic neurons present in the caudate-putamen.

Analogs of NGF fragments with NGF activity as described above have potential pharmaceutical applications in situations involving nerve damage from traumatic accidents, stroke and encephalitis.

The peptides of the invention may be administered by parenteral means, including subcutaneous and intramuscular injection, injection into the CNS, implantation of sustained-release depots, intravenous injection, intranasal administration, and the like. Because the

present peptides have the potential of being metabolically stable and of crossing the blood-brain barrier, they have the potential of being administered orally or by intravenous injection.

The NGF peptides may be administered as a pharmaceutical composition comprising the peptide in combination with a pharmaceutically acceptable excipient. Such compositions may be aqueous solutions, emulsions, suspensions, gels, liposomal suspensions, and the like. Thus, suitable excipients include water, saline, Ringer's solution, dextrose solution, and solutions of ethanol, glucose, sucrose, dextran, mannose, mannitol, sorbitol, polyethylene glycol (PEG), phosphate, acetate, gelatin, collagen, and the like. One may additionally include suitable preservatives, stabilizers, antioxidants, antimicrobials, buffering agents and the like. A thorough discussion of pharmaceutically acceptable excipients is available in Remington's Pharmaceutical Sciences, 2nd Ed., Mack Publishing Co.

Alternatively, one may incorporate or encapsulate the NGF peptides in a suitable polymer matrix, liposome or membrane, thus providing a sustained-release delivery device suitable for implantation near the site to be treated locally. In general, with sustained release delivery, the formulations are constructed so as to achieve a constant concentration which will be bioequivalent to about 100 times the serum level of NGF or 10 times the tissue concentration.

The amount of the peptide required to treat any particular neural disorder will of course vary depending upon the nature and severity of the disorder, the age and condition of the subject, and other factors readily determined by one of ordinary skill in the art. Suitable dosages are from 10 μg/kg to 1000 mg/kg, more preferably 10 mg/kg to 400 mg/kg.

As appreciated by one skilled in the art, the concentration of the formulation for NGF antagonists is generally 10-fold higher, regardless of the mode of administration. The higher dosage assures that the NGF inhibitor is able to compete effectively with endogenously produced NGF.

The following examples are presented in further illustration, but not limitation, of the invention.

Examples

NGF Peptides

A. Preparation of NGF

NGF was purified from male mouse submaxillary gland and characterized as previously described (Mobley et al, (1976) Biochem 15(25):5543-5551).

B. Synthesis and Purification of Peptides

Amide-form peptides were synthesized corresponding to subregions of the NGF molecule. These peptides are listed in Table 3 and are found in regions corresponding to residues 28-38, 66-75 and 98-106. It was speculated that the region from residues 28-38, 66-75 and 98-106, are directly involved in receptor interaction because of their relatively high degree of hydrophilicity, cross-species conservation, and in the case of Regions A and C, the probability of containing a β-turn. Therefore, a synthetic peptide modeled from these regions might serve as an NGF antagonist or agonist. The peptides shown in Table 3 were prepared on an ABI 430A peptide synthesizer using 0.5 millimoles of p-methyl-benzhydrylamine resin (ABI) and Boc amino acid derivatives (Peninsula Laboratories, San Carlos, CA) with standard solvents and reagents. Peptides were cleaved from their resins with anhydrous hydrogen fluoride in the presence of anisole and

dimethylsulfide at 0°C. Cleaved peptides were washed with ether (3 x 50 ml), extracted with 10% acetic acid, lyophilized, redissolved in 10 ml purified H_2O and lyophilized. The C-terminus of the peptides thus produced ended with an amide. Peptides were purified by high pressure liquid chromatography using either: (1) a C18 uBondapak column (Waters and Co.), eluted with a linear gradient of acetonitrile in 0.1% trifluoracetic acid; and/or (2) a polysulfoethyl A cation exchange column (Poly-LC, Columbia, MD) employing a gradient of 20 to 500 mM ammonium acetate in 25% acetonitrile, pH 6.8. The central portions of the major peak (230 nm) from each of several runs were combined. An aliquot was reapplied to the column under the same conditions. The analytical runs showed no detectable impurities. Peptide composition was confirmed by quantitative amino acid analysis. The sequence of peptide C5 (KGKE) was determined via automated Edman degradation.

TABLE 3
Activity of NGF Peptides

	Putative Receptor Binding Domain Region		IC_{50}** (μ moles/ml) Antagonist Activity	Lowest Concentration for Detected Agonist Activity (μ moles/ml)
C1	ATDIKGKEVTV-NH_2		>0.500	ND*
C2	DIKGKEVTV-NH_2		>0.500	0.500
C3	KGKEVTV-NH_2		0.450	NA†
C4	DIKGKE-NH_2		0.500	ND
C5	KGKE-NH_2		0.095	ND
C6	DIKG-NH_2		0.300	ND
C7	KGK-NH_2		0.125	ND
C8	IKG-NH_2		1.060	ND
C9	IKGK-NH_2		0.710	ND
C10	ATDIKG-NH_2		>1.0	ND
C12	GKE-NH_2		ND	ND
C13	KG-NH_2		ND	ND
A1	SGCRGIDSKH-NH_2		>1.000	ND
A2	RGIDSKH-NH_2		>1.000	ND
A3	RGIDS-NH_2		ND	ND
A4	RGIDSK-NH_2		ND	ND
B1	AWRFIRIDT-NH_2		1.150	ND
B2	RIDT-NH_2		0.700	ND
B3	RFIRIDT-NH_2		ND	ND
B4	RFIRID-NH_2		ND	ND
Retest Values				
C1			1.100	ND
C2			1.100	0.500
C3			ND	1.000
C5			0.200	ND

C. Bioassay With Dorsal Root Ganglia Neurons

NGF bioassays using dissociated embryonic chick dorsal root ganglia neurons have been described (Varon et al, (1981) Dev Brain Res 1:73-87); Riopelle et al, (1981) J Neurobiol 12:175-186). Approximately 30 dorsal root ganglia were dissected from each developmental day 8 chick

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* ND = Not determined

† NA = No activity up to .500 concentration

**IC_{50} = Concentration at which 50% reduction in neurite-bearing neurons is observed.

embryo. Ganglia were incubated for 10 min at 37°C in 5 ml of calcium-magnesium free balanced salt solution (CMF) and then for an additional 10 min in 0.01% trypsin in CMF. Ganglia were then washed twice with 5 ml 10% fetal calf serum in HAMS F-12 medium supplemented with Hanks balanced salt solution (culture medium) and dissociated in 1.5 ml culture medium by trituration (10-15 times) through a flamed glass pipet. The resulting cell suspension was diluted to 10 ml in culture medium, added to a 100 mm culture dish and incubated for 2 hours at 37°C in 7% CO_2 - 93% air. During this incubation, non-neurons more readily attach to the substratum; therefore, the resulting supernatant contained a relatively enriched neuronal population (80-90% neurons). Each ganglion yielded 15,000-20,000 neurons; more than 95% of which excluded trypan blue.

Tissue culture wells (96 well tissue culture plates, A/2 Costar) were precoated with 0.1 mg/ml of polyornithine for 1-2 hours and then washed twice with 100 ul of sterile water. To each well was added 25 µl of NGF and 25 µl of peptide, each in various dilutions (as indicated in Figures 1B, 2 and 3) of culture media and 50 ul of a cell suspension diluted with culture media to a density of 100,000 cells/ml (5000 cells/well). In a second experiment, 2 µg/ml of polyornithine were used to coat the wells, and 12.5 µl each of NGF and the peptide to be tested were added. Additionally, 25 µl of the diluted cell suspension at the same density (e.g., 100,000 cells/ml (2500 cells/well)) were added.

The results obtained were similar in each experiment; Figure 1A represents the data obtained from the second experiment. Cultures were incubated for 24 hr at 37°C in 93% air - 7% CO_2. Neurite outgrowth was assessed by phase contrast microscopy. The number of neurite-bearing cells in a fixed area of the well was counted. In selected cultures, neuronal survival may be assessed by the addition of MTT (3-(4,5-dimethylthiazol-2-

yl)-2,5-diphenyl tetrazolium bromide, Sigma, St. Louis). Blue cellular staining is indicative of ongoing mitochondrial function. Maximum neurite outgrowth was obtained at approximately 800 pg/ml NGF.

Table 3 provides the results for the individual peptides that were tested for agonist or antagonist potential. As observed from the results, peptides from each of the three putative receptor binding domains exhibited some degree of antagonist activity.

The concentration of peptide causing a 50% reduction in the number of neurite-bearing neurons (IC_{50}) was determined as shown in Table 3. The inhibition of neurite outgrowth in the presence of C5, C1, or C2 supported by a fixed concentration of NGF, is shown in Figure 1A. As shown therein, the number of neurite-bearing neurons was reduced with C1 and C2 but to a lesser extent than that observed for C5.

As demonstrated in Figure 1B, the blocking effect of C5 could be completely overcome by increased concentrations of NGF. The parallel displacement of the dose-response curve suggests that the inhibition by C5 is competitive. The equilibrium dissociation constant for C5 was determined from the formula for antagonist inhibition of agonist stimulation (Furchgott, (1967) Ann NY Acad Sci 139:553-570): $K_i = B/((A'/A)-1)$ where B is the concentration of C5 and A' and A are the EC_{50} values of the agonist dose response curves in the presence and absence of C5. The K_i for C5 was calculated at 47.8 μ M.

Potential nonspecific toxicity of peptide C5 (KGKE) was investigated with embryologic day 10 dorsal root ganglia neurons which also extend neurites in the presence of ciliary neurotrophic factor (CNTF). The effect of peptide C5 on NGF-independent neurite outgrowth was assessed.

C5 (up to 2.6 μ moles/ml) had no detectable effect on the number of embryologic day 10 dorsal root

ganglia neurons bearing neurites in response to CNTF (Figure 3).

D. Support of Neuronal Survival by Synthetic Peptides

Neurons were cultured in the presence of peptides without NGF. Peptide C2 demonstrated neurite-promoting activity. As shown in Figure 2, the trophic effect of C2 was not equal to NGF at 1μ moles/ml; C2 elicited only 35% of the effect produced by NGF.

E. Resistance to Proteolysis

The resistance of peptide C5 to proteolytic cleavage during cell culture was assessed. Quadruplicate wells were prepared each containing 2.0μ mole/ml of peptide in culture medium, 100 pg/ml NGF and cells as described above. After 24 hr in culture, 320 ul (4 wells x 80 ul) of culture medium were collected. Fresh culture medium containing 2.0 μmole/ml of peptide was also prepared. Both samples of peptide-containing medium were filtered through a Centricon membrane (Amicon, Bedford) with a molecular weight cutoff of 10,000. 250 μl of filtrate was loaded onto a C18 uBondapak analytic HPLC column and run under the same conditions described above for peptide purification. The quantity of peptides in fresh compared to post-culture medium was determined by integration of their absorbance peaks. After 24 hr in culture, more than 80-85% of the initial quantity of peptide C5 was recovered.

F. Ligand Binding Assays

NGF was radiolabeled with ^{125}I to a specific radioactivity of 40-60 cpm/pg using lactoperoxidase (Sutter et al, (1979) J Biol Chem 254:5972-5982). The radioactivity was greater than 90% precipitable in 10% TCA. In preliminary experiments, binding of ^{125}I-NGF at 37°C reached a maximum at 30-45 min.

The direct binding assay for ^{125}I-NGF has been described by Sutter et al (1979, supra) and is incorporated herein by reference. Steady state binding was determined in incubations of dorsal root ganglia cells and ^{125}I-NGF in PBS containing serum albumin (1 mg/ml) at 37°C for 60 min. Bound and free ^{125}I-NGF were separated by centrifugation of the incubation mixture for 2 min at 10,000 x g. After the supernatant was aspirated the tips of the incubation tubes were cut and counted in a Beckman 300 Gamma counter.

Figure 4 demonstrates that at a concentration of 1000 nmoles/ml (n=8), and 2000 nmoles/ml (n=4) peptide C5 produced no change in the specific binding of ^{125}I-NGF at 10 pM.

NGF Regulation of Gene Expression

A. Tissue Preparation

The birth of hamster litters was recorded as day zero. Hamsters were sacrificed at various postnatal ages and several brain regions were quickly dissected. The regions sampled included septum (basal forebrain), caudate-putamen, thalamus, hippocampus, frontolateral neocortex, and brainstem. Dissections were performed as described (Johnston et al, (1987) Neurochem Res 12:985-994). Subiculum was included with hippocampus. Caudal midbrain plus pons constituted the brainstem. For prenatal determinations, a timed-pregnancy animal was anesthetized with Nembutal (15 mg, i.p.) and individual embryos were removed and dissected. Whole forebrain was taken in place of frontolateral neocortex in prenatal animals. ChAT activity was determined in selected tissues as described (Mobley et al, (1985) Science 229:284-287).

B. NGF Preparation and Treatment

NGF was prepared by ion-exchange chromatography and characterized as described in the first example. In experiments in which multiple injections were given, NGF (30 µg) was administered intraventricularly (Mobley et al, (1985) supra) on postnatal day (PD) 3, 5 and 7 and sacrifice was on PD 9. Vehicle-injected animals served as controls. In experiments in which a single injection was given, NGF (30 µg) was administered intraventricularly on PD 7 and animals were sacrificed on PD 9. Controls included animals treated with NGF (30 µg) denatured by reduction and carbamoylmethylation (Morris et al, (1971) Neurobiology 1:64-67), or injection vehicle alone.

C. RNA Preparation and Blotting

Total RNA was prepared as taught in Kitaguchi et al, (1988) Nature 331:530-532). For slot hybridization, RNA samples (5 µg) were ethanol precipitated, vacuum dried, dissolved in 50 µl sterile water and incubated with 150 µl of 6.1 M formaldehyde in 10 x SSC at 65°C for 15 min. Nitrocellulose (BA 85; Schleicher and Schuell) was prewetted with 10 x SSC and placed on a slot minifold apparatus (Schleicher and Schuell). Samples were loaded and vacuum applied (Kafatos et al, (1979) Nucl Acid Res 7:1541-1552). Filters were baked at 80°C for 2 hr. The DNA insert of pHaPrP (Oesch et al, (1985) Cell 40:735-746) was excised with BamHI (Boehringer-Mannheim) and isolated by preparative gel electrophoresis.

The recovered fragment was labeled to a specific activity of 10^9 dpm/µg using random priming oligonucleotides (P.L. Biochemicals) and the Klenow fragment (Boehringer-Mannheim). Prehybridization and hybridization of the filters were performed using conventional procedures. Hybridizations were carried out for 16 hr at 42°C in 3 x SSC, 50% formamide, 0.05 M HEPES (pH 7.4), 0.2 mg/ml salmon sperm DNA, 0.15 mg/ml yeast RNA, 0.02% bovine

serum albumin, and 0.02% polyvinyl pyrrolidone. Filters were washed in 0.1 x SSC, 0.1% SDS at 60°C for 3 hr and autoradiographed for 1-3 days at -70°C using Dupont Cronex intensifying screens and Kodak XAR-5 film. Autoradiograms were densitometrically scanned (LKB 2202 Ultroscan; 3390 A Hewlett-Packard integrator) to assess the amount of prion protein (PrP) or β-amyloid precursor protein (BAPP) mRNA. Measurements were accepted when they fell in the range through which signal intensity was linearly related to the amount of RNA loaded.

In examining the developmental time course for PrP gene expression, the tissues of 4 animals were pooled for the prenatal measurement of forebrain PrP mRNA; 7 animals were pooled for brainstem. For each postnatal sample, RNA was prepared from pooled brain regions of entire hamster litters or from 3 to 5 adults. To examine the postnatal ontogenesis of β-amyloid precursor protein mRNA, RNA was prepared as indicated above and two separate samples were examined at each postnatal age.

The relative levels of PrP mRNA in several brain regions was determined in the adult. Individual samples (n=2 for each) were compared to a standard curve prepared by blotting known amounts of PrP cDNA. The cDNA insert of pHaPrP was ethanol precipitated, the pellets dried and resuspended in 400 µl of TE buffer. Serial 10-fold dilutions were then prepared. After the addition of one-tenth volume 3 N NaOH, the dilutions were incubated at 65°C for 30 min, chilled, and following the addition of 400 µl of 2 M ammonium acetate, were loaded under vacuum onto nitrocellulose prewetted with 1 M ammonium acetate (Kafatos et al, (1979) supra). Filters were hybridized as described above.

For Northern blotting analysis, RNA samples were ethanol precipitated, washed once with 70% ethanol, and dissolved in 50% formamide, 2.2 M formaldehyde. After the addition of one-fifth volume of 10% Ficoll with 1%

bromphenol blue, samples were electrophoresed through 1% agarose. The running buffer was 20 mM MOPs (pH 7.0), 1 mM EDTA, 2.2 M formaldehyde. After electrophoresis, gels were soaked in 20 x SSC and the RNA transferred to nitrocellulose as described (Thomas, (1980) Proc Natl Acad Sci USA 72:5201-5205). Filters were prehybridized and hybridized as indicated above.

D. Prion Protein Measurement

PrP levels were measured in the septum of NGF- and vehicle-injected hamsters following a single treatment on PD7. Each tissue sample (the septum of an individual animal) was weighed and then disrupted by trituration through a Pasteur pipette in cold Tris-NaCl-EDTA buffer containing 0.15% NP40 and 0.15% DOC. Extraction was performed on ice for 5 min; cell debris was pelleted 2,000 x g for 5 min and proteins were precipitated in 80% methanol at -20^{o}C for 24 hr. Electrophoresis was performed in 12% polyacrylamide gels according to the method of Laemmli (Laemmli, (1970) Nature 227:680-685). Proteins were electroblotted to nitrocellulose filter paper (Towbin et al, (1979) Proc Natl Acad Sci USA 76:4350-4354). The filter was blocked with Blotto (5% nonfat-dry milk in PBS), incubated at room temperature with primary antisera (monoclonal antibody 13A5, raised against hamster PrP, (Barry et al, (1986) J Infect Dis 154:518-521), and immunostained with the Protoblot alkaline phosphatase system (Promega Biotech, Madison, WI). Nitrocellulose filters were densitometrically scanned to determine the amount of PrP.

Results

Developmental Regulation of PrP Gene Expression

To investigate the regulation of PrP gene expression, PrP mRNA was measured in several regions of the hamster brain during pre- and postnatal development. While PrP mRNA was found in all tissues sampled, its ontogenesis varied regionally. Three developmental patterns for PrP gene expression were found: (i) early expression, observed in brainstem and neocortex; (ii) intermediate expression, seen in hippocampus, thalamus and caudate-putamen; and (iii) delayed expression, found in septum. Low levels of PrP mRNA were detected in the brainstem in the immediate prenatal period, but increased rapidly to near-adult levels during the first postnatal week. PrP gene expression in neocortex was similar to brainstem in its developmental pattern except that greater than adult levels were seen during the second postnatal week. In contrast, there was little or no measurable PrP mRNA in hippocampus, caudate-putamen or thalamus through postnatal day 3. By 6 days PrP mRNA had increased. At this time PrP mRNA was 40% of adult levels in hippocampus, 97% in thalamus and 124% in caudate-putamen. PrP mRNA rose to adult or greater than adult levels in all three regions over the next two weeks. In caudate-putamen levels 220% of those in the adult were found at PD 16. Individual brain regions also differed in the amount of PrP mRNA found in adult animals. Randomly selected postnatal RNA samples were also analyzed by Northern analysis. In all instances the hybridizing species migrated as a 2.1 kb band.

Septum was clearly delayed in its expression of PrP mRNA. Not until 9 days of age was PrP mRNA detected in this tissue; 17% of the adult level was measured at this time. PrP mRNA levels rose to 60-70% of adult values by 12 days of age and reached maximal values by PD 16.

The delayed appearance of PrP mRNA in septum exhibited a pattern reminiscent of the time-course followed by septo-hippocampal cholinergic markers in the rat (Large et al, (1986) Science 234:352-355; Shelton et al, (1979) Brain Res 163:263-275). To determine if cholinergic markers followed the same pattern in the hamster, we measured the activity of ChAT, the enzyme which catalyzes the final step of acetylcholine synthesis and is selectively localized in cholinergic neurons. ChAT activity was measured at intervals during postnatal development and the data were compared to those for PrP mRNA. ChAT specific activity in septum was less than 20% of adult levels at PD 3 and remained low through 9 days of age rising to adult levels over the ensuing week. From PD 9 through 16, the developmental increase in ChAT activity in the septum was virtually coincident with that for PrP mRNA. Thus, increasing PrP gene expression in the developing hamster septum was temporally coincident with the differentiation of cholinergic neurons.

NGF Effects on PrP Gene Expression

The coincident rises of ChAT activity and PrP mRNA raised the possibility that these markers were under coordinate control during the development of septal cholinergic neurons. One approach to defining the relationship between PrP gene expression and the development of cholinergic neurons was suggested by prior studies with NGF. In rat brain, NGF injections selectively increased septal ChAT activity but had no effect on glutamic acid decarboxylase or tyrosine hydroxylase, neurotransmitter enzyme markers for GABA-ergic and catecholaminergic neurons, respectively. Moreover, NGF receptors in the rat septum are localized to neuronal cell bodies which in their size and distribution closely resemble cholinergic neurons. Selectivity of NGF action thereby provided a means for examining neuro-chemical events in

developing cholinergic neurons. To determine whether or not NGF influences the expression of PrP mRNA, NGF (30 µg) was administered intraventricularly to neonatal hamsters on PD 3, 5, and 7 and the animals were sacrificed on PD 9. Control littermates received injection vehicle alone. In normal animals, ChAT activity at day 9 was 34% of adult levels and PrP mRNA was 17% of adult levels. Similar values were found in vehicle-injected animals. In NGF-treated animals, ChAT activity was increased almost two-fold (Figure 5A), indicating that hamster basal forebrain cholinergic neurons responded to NGF injection. NGF induced a much larger change in PrP mRNA. The level of septal PrP mRNA was increased approximately 10-fold relative to controls and exceeded the levels found in adults.

In a prior study, we found that a single injection of NGF elicited an increase in the neurochemical differentiation of developing forebrain cholinergic neurons. The NGF-mediated stimulation of ChAT activity in the septum of rat neonates was first evident 48 hr after a single intraventricular injection and persisted for the next several days (Johnston et al, (1987) supra). To determine whether or not an effect of NGF on PrP mRNA would be demonstrable over this time course, NGF was given as a single (30 µg) injection to neonatal hamsters at day 7 and animals were sacrificed 48 hours later, on day 9. Septal ChAT activity was increased 100% relative to vehicle-injected animals [mean ± SEM; vehicle-injected = 22.3 ± 1.3 nmol acetylcholine (ACh) formed/hr/mg protein (n=4); NGF = 44.0 ± 5.0 (n=4); $P < 0.005$, Student's t-test]; PrP mRNA levels were increased nearly 9-fold (Figure 5B). Northern blot analysis showed that the PrP mRNA induced by NGF treatment migrated identically to that found in vehicle-injected animals and adults (Figure 5C). No increase in ChAT activity or PrP mRNA was produced by

injection of NGF denatured by reduction and carbamoylmethylation.

The increase in PrP mRNA induced by NGF treatment was confined to specific brain regions. Of note, the caudate-putamen in the rat contains NGF-responsive cholinergic neurons which are distinct from those found in basal forebrain. A single NGF injection in neonatal hamsters increased ChAT activity in caudate-putamen by approximately 65% [vehicle = 56.1 $\pm$ 4.5 (n=3); NGF = 92.4 $\pm$ 6.3 (n=3); $P > 0.01$, Student's t-test]. PrP mRNA was increased by 70% relative to vehicle-injected animals (Figure 5B). In contrast to the responses in septum and caudate-putamen, NGF had no effect on PrP mRNA levels in thalamus or hippocampus (Figure 5B).

NGF Effects on the Levels of PrP

NGF effects on basal forebrain neurons produced a far greater increase in PrP mRNA levels than in ChAT activity in either the multiple or single injection protocol. To compare NGF effects on PrP mRNA levels with those for the prion protein, we measured the level of PrP in the septum 48 hr following a single NGF (30 ug) injection given on PD 7. By Western blot analysis there was a 3.3-fold increase in PrP. Thus, under the conditions of this experiment, the NGF-induced increase in the level of the PrP protein was somewhat less marked than the increase in PrP mRNA.

Regulation of ß-Protein Precursor (ß-PP) Gene Expression in Developing Septum; NGF Effects

As was the case for PrP, ß-PP mRNA was found in all the brain tissues sampled. The size of the mRNA in septum was 3.5 kb. The pattern for the ontogenesis of ß-PP mRNA levels in the septum was similar to that for PrP. ß-PP mRNA levels were first detected at PD9 when they equaled 19% of those found in the adult. There was

an increase in β-PP mRNA during the next several days such that peak levels (325% of adult) were achieved at PD16.

NGF injection had a marked effect on the level of β-PP mRNA. After a single treatment on PD7, mRNA levels for β-PP were increased 4-fold relative to vehicle-injected animals. As shown in Figure 6, the effect of NGF was regionally specific in that NGF induced no increase in β-PP in the thalamus.

What is Claimed is:

1. A peptide having the ability to induce a nerve growth factor (NGF)-associated biological response in rat dorsal root ganglia, wherein greater than 50% of the amino acid sequence of the peptide is at least 20% homologous with a receptor binding domain of NGF said peptide being preferably of mammalian origin, especially of human origin.

2. A peptide having the ability to induce a nerve growth factor (NGF)-associated biological response in rat dorsal root ganglia, having from 3 to 28 amino acid residues substantially homologous to a portion of the region defined by residues 20-48 of NGF, numbered in accordance with native, murine NGF, especially 3 to 11 amino acid residues substantially homologous to residues 28-38 of NGF, particularly with the α-carboxyl group of the residue at the C-terminus amidated.

3. The peptide of claim 2 which is selected from the group consisting of Ala-Thr-Asp-Ile-Lys-Gly-Glu-Val-Thr-Val-NH_2, Lys-Glu-Val-Thr-Val-NH_2, Asp-Ile-Lys-Gly-Lys-Glu-Val-Thr-Val-NH_2, Lys-Gly-Lys-Glu-Val-Thr-Val-NH_2, Lys-Gly-Lys-Glu-Val-Thr-Val-NH_2, Ala-Thr-Asp-Ile-Lys-Gly-NH_2, Asp-Ile-Lys-Gly-Lys-Glu-NH_2, Lys-Gly-Lys-Glu-NH_2, Asp-Ile-Lys-Gly-NH_2, Ile-Lys-Gly-Lys-NH_2, Ile-Lys-Gly-NH_2, Gly-Lys-Glu-Val-NH_2, Gly-Lys-Glu-NH_2, Lys-Glu-NH_2 and Lys-Gly-Lys-NH_2.

4. The peptide of claim 2 having the formula:
R_1-AA_1-AA_2-AA_3-AA_4-R_2 wherein:
AA_1 is an amino acid residue selected from the group consisting of Lys, d-Lys, Arg, d-Arg, Pro, d-Pro, Nle, homo-Arg, d-homo-Arg, Orn, and d-Orn or is deleted;

AA_2 is an amino acid residue selected from the group consisting of Gly, d-Gly, Pro, d-Pro, Ala, Aib, d-Ala, Val, d-Val, Leu, d-Leu, Ile, and d-Ile or is deleted;

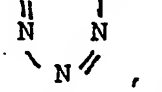
AA_3 is an amino acid residue selected from the group consisting of Lys, d-Lys, Arg, d-Arg, Pro, d-Pro, and Nle or is deleted;

AA_4 either is a residue of an amino acid selected from the group consisting of Glu, d-Glu, Gln, d-Gln, Asp, d-Asp, Asn, d-Asn, Phe, and d-Phe, or together with R_2 forms $-NH-\underset{CH_2COOH}{CH}-R_4$ or $-NH-\underset{(CH_2)_2COOH}{CH}-R_4$, or is deleted;

R_1 is attached to the amino group of AA_1 and selected from the group consisting of hydrogen, lower alkyl, lower alkyl-carbonyl, lower alkenyl, lower alkynyl, formyl, aryl, aroyl, aryloxy-carbonyl, aralkyloxy-carbonyl, lower alkyloxy-carbonyl, benzoyl, 1- or 2-thenoyl, nicotinoyl, dihydronicotinoyl, N-alkyldihydronicotinoyl, isonicotinoyl, N-alkyldihydroisonicotinoyl, tetrahydronicotinoyl, and N-alkyltetrahydronicotinoyl;

R_2 is attached to the carbonyl group carbon of AA_4 and selected from the group consisting of OH, NH_2, OR_3, OR_3OH, and NHR_3;

R_3 is selected from the group consisting of lower alkyl and aryl groups; and

R_4 is selected from the group consisting of PO_3H_2, $B(OH)_2$, CH_2OH, SO_3H, and  , especially Lys-Gly-Lys-Glu, having an amidated C-terminus.

5. The peptide of claim 2 having the formula:

$R_1-AA_1,-AA_2-AA_3-AA_4-AA_5-AA_6-AA_7-AA_8-AA_9-R_2$

wherein:

AA_1 is an amino acid selected from the group consisting of Asp, d-Asp, Glu, d-Glu, Asn, d-Asn, Gln, d-Gln, and pyro-Glu, or is deleted;

AA_2 is an amino acid selected from the group consisting of Ile, d-Ile, Val, d-Val, AdaA, AdaG, Leu, d-Leu, Met, and d-Met, or is deleted;

AA_3 is an amino acid selected from the group consisting of Lys, d-Lys, Arg, d-Arg, Ile, d-Ile, Met, homo-Arg, d-homo-Arg, Orn, and d-Orn, or is deleted;

AA_4 is an amino acid selected from the group consisting of Gly, Ala, d-Ala, Pro, d-Pro, Aib, β-Ala, and Acp, or is deleted;

AA_5 is an amino acid selected from the group consisting of Lys, d-Lys, Arg, homo-Arg, d-homo-Arg, Met, Ile, d-Ile, Orn, and d-Orn, or is deleted;

AA_6 is an amino acid selected from the group consisting of Glu, d-Glu, Gln, d-Gln, Asn, Asp, d-Asp, and d-Asn, or is deleted;

AA_7 is an amino acid selected from the group consisting of Val, d-Val, Leu, d-Leu, Ile, d-Ile, Met, Chg, Cha, and d-Met, or is deleted;

AA_8 is an amino acid selected from the group consisting of Thr, d-Thr, allo-Thr, Ser, d-Ser, Met, d-Met, Met(O), and d-Met(O), or is deleted;

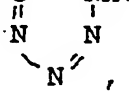
AA_9 either is a residue of an amino acid selected from the group consisting of Val, d-Val, Leu, d-Leu, Chg, and Valinol, or together with R_2 forms $-NH-CH-R_4$ or $-NH-CH-R_4$ or is deleted;
CH_2COOH $(CH_2)_2COOH$

R_1 is attached to the amino group of AA_1 and selected from the group consisting of hydrogen, lower alkyl, lower alkyl-carbonyl, lower alkenyl, lower alkynyl, formyl, aryl, aroyl, aryloxy-carbonyl, aralkyloxy-carbonyl, lower alkyloxycarbonyl, benzoyl, 1- or 2-thenoyl, nicotinoyl, dihydronicotinoyl, N-alkyldihydronicotinoyl, isonicotinoyl,

N-alkyldihydroisonicotinoyl, tetrahydronicotinoyl, and N-alkyltetrahydronicotinoyl;

R_2 is attached to the carbonyl group carbon of AA_9 and selected from the group consisting of OH, NH_2, OR_3, OR_3OH, and NHR_3;

R_3 is selected from the group consisting of lower alkyl and aryl groups; and

R_4 is selected from the group consisting of PO_3H_2, $B(OH)_2$, CH_2OH, SO_3H, and  , especially wherein AA_1 is Asp, AA_2 is Ile, AA_3 is Lys, AA_4 is Gly, AA_5 is Lys, AA_6 is Glu, AA_7 is Val, AA_8 is Thr, AA_9 is Val, R_1 is H and R_2 is NH_2.

6. The peptide of claim 4 or 5 wherein AA_1 is selected from the group consisting of Lys, d-Lys, Orn and d-Orn and R_1 is other than hydrogen.

7. A peptide having the ability to induce a nerve growth factor (NGF)-associated biological response in rat dorsal root ganglia, having from 3 to 34 amino acid residues substantially homologous to residues 50-84 of NGF, numbered in accordance with native, murine NGF, especially from 3 to 10 amino acid residues substantially homologous to a portion of the region defined by residues 66-75 of NGF, particularly with the αcarboxyl group of the residue at the C-terminus amidated.

8. The peptide of claim 7 which is Ser-Gly-Cys-Arg-Gly-Ile-Asp-Ser-Lys-His-NH_2 and Arg-Gly-Ile-Asp-Ser-Lys-His-NH_2.

9. A peptide having the ability to induce a nerve growth factor (NGF)-associated biological response in rat dorsal root ganglia, having from 3 to 25 amino acid

residues substantially homologous to residues 87-112 of NGF, numbered in accordance with native, murine NGF, especially from 3 to 9 amino acid residues substantially homologous to residues 98-106 of NGF, particularly with the α-carboxyl group of the residue at the C-terminus amidated.

10. The peptide of claim 9 which is Ala-Trp-Arg-Phe-Ile-Arg-Ile-Asp-Thr-NH_2 or Arg-Ile-Asp-Thr-NH_2.

11. The peptide of claim 1 which is selected from the group consisting of

Lys-Gly-Lys-Glutamic Acid,

Lys-Gly-Lys-NH-CH(CH_2CH_2COOH)-CH_2OH,

Lys-Gly-Lys-Glu-α-NH-CH_3,

Lys-Gly-Lys-Gln-OH,

Lys-Gly-Lys-Gln-NH_2,

Lys-Gly-Lys-d-Glu-NH_2,

Lys-Gly-Lys-CH(CH_2CH_2COOH)-PO_3H_2,

Lys-Gly-Lys-Gln-NH_2,

Lys-Gly-Lys-NH-CH(CH_2CH_2-COOH)-CH_2OH

Lys-Gly-Lys-NH-CH(CH_2CH_2-CH_2OH)-CH_2OH

Lys-Gly-Lys-NH-CH(CH_2CH_2-CH_2OH)-CH_2CONH_2

Lys-Gly-Lys-Glu-NH_2

Lys-Gly-Lys-Phe-NH_2

(1-methyl-1,4-dihydronicotinoyl)
Lys-Gly-Lys-Gln-NH_2
Lys-Gly-Arg-Glu-NH_2,
Lys-Gly-Arg-Glu-NH-CH_3,
Lys-Gly-Arg-Gln-OH,
Lys-Gly-Nle-Glu-NH_2,
Lys-Gly-Pro-Glu-NH_2,
Lys-Gly-Pro-NH-CH(CH_2CH_2COOH)-PO_3H_2,
Lys-Pro-Lys-Asp-NH-CH_3,
Lys-Ala-Lys-Glu-NH-CH_3,
Lys-Val-Lys-Glu-NH_2
Lys-Pro-Lys-Glu-NH-CH_3,
Lys-d-Ala-Lys-Glu-NH_2,
Lys-Ala-Lys-Glu-NH_2,
Lys-d-Ala-Lys-Glu-NH_2,
Lys-Aib-Lys-Glu-NH_2,
Lys-Leu-Lys-Glu-NH_2
Orn-Gly-Lys-Glu-NH_2
d-Orn-Gly-Lys-Glu-NH_2
d-Lys-Gly-Lys-Glu-NH_2,
d-Lys-d-Ala-Lys-Glu-NH_2,
d-Lys-Gly-Lys-Glu-NH-CH_3,
Arg-Gly-Lys-Glu-NH_2,
d-Arg-Gly-Lys-Glu-NH_2,
Arg-Gly-Lys-Glu-NH-CH_3,
Arg-Gly-Arg-Glu-NH_2,
d-Lys-Gly-Arg-Asp-NH_2,
homo-Arg-Gly-Lys-Glu-NH_2
d-homo-Arg-Gly-Lys-Glu-NH_2
Lys-Gly-Lys-Glu-Lys-Gly-Lys-Glu-NH_2,
Lys-Gly-Lys-Glu-β-Ala-Lys-Gly-Lys-Glu-NH_2,
the polymeric peptides
(Lys-Gly-Lys-Glu(NH_2))$_n$-R_2,
(Lys-Gly-Lys-Glu)$_n$-R_2,

$(Lys\text{-}Gly\text{-}Lys\text{-}Glu\text{-}Gly)_n\text{-}R_2$,
$(Lys\text{-}Gly\text{-}Lys\text{-}Glu\text{-}\beta\text{-}Ala)_n\text{-}R_2$, and
$(Lys\text{-}Gly\text{-}Lys\text{-}Glu\text{-}Pro)_n\text{-}R_2$, wherein n is an integer from 2 to 40,

Cyclo(-Lys-Gly-Lys-Glu-Lys-Gly-Lys-Glu-),

Cyclo(-Lys-Gly-Lys-Glu-Pro-Lys-Gly-Lys-Glu-Pro-),

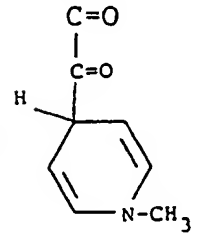
Cyclo(-Lys-Gly-Lys-Glu-β-Ala-Lys-Gly-Lys-Glu-β-Ala-),

$Lys(Ac)\text{-}Gly\text{-}Lys\text{-}Glu\text{-}NH_2$

$Lys\text{-}Gly\text{-}Lys(Ac)\text{-}Glu\text{-}NH_2$

$Lys\text{-}Gly\text{-}Lys(Bz)\text{-}Glu\text{-}NH_2$

$Lys\text{-}Gly\text{-}Lys\text{-}Glu\text{-}NH_2$



Lys-Gly-Lys-Glu-(Acp)-R_2,

Lys-Gly-Lys-Glu-(Acp)-NH_2,

Lys-Gly-Lys-Glu-NH-$(CH_2)_6$-OH,

Lys-Ala-Lys-Glu-Acp-OH,

Lys-Pro-Lys-Glu-Acp-OH,

Lys-Ala-Lys-Glu-Acp-NH_2,

Lys-Pro-Lys-Glu-Acp-NH_2,

Lys-Pro-Lys-Glu-$NH(CH_2)_6$-OH,

d-Asp-Ile-Lys-Gly-Lys-Glu-Val-Thr-Val-R_2,

d-Asp-Ile-Lys-Gly-Lys-Glu-Val-Thr-Val-OH,

d-Asp-Ile-Lys-Gly-Lys-Glu-Val-Thr-Val-OCH_3,

d-Asp-Ile-Lys-Gly-Lys-Glu-Val-Thr-Val-NH_2,

d-Asp-Ile-Lys-Gly-Lys-Glu-Val-Thr-Val-NH-CH_3,

d-Asp-Ile-Lys-Gly-Lys-Glu-Val-Thr-Val-PO_3H_2,

Glu-Ile-Lys-Gly-Lys-Glu-Val-Thr-Val-R_2,

Asp-Ile-Lys-Gly-Lys-Glu-Val-Thr-Val-R_2,

(1-methyl-1,4-dihydronicotinoyl)

Asn-Ile-Lys-Gly-Lys-Glu-Val-Thr-Val-NH_2

Gln-Ile-Lys-Gly-Lys-Glu-Val-Thr-Val-R_2,

d-Glu-Ile-Lys-Gly-Lys-Glu-Val-Thr-Val-R_2,

d-Gln-Ile-Lys-Gly-Lys-Glu-Val-Thr-Val-R_2,

Asp-Leu-Lys-Gly-Lys-Glu-Val-Thr-Val-R_2,

Asp-Val-Lys-Gly-Lys-Glu-Val-Thr-Val-R_2,

Asp-AdaA-Lys-Gly-Lys-Glu-Val-Thr-Val-R_2,

Asp-AdaG-Lys-Gly-Lys-Glu-Val-Thr-Val-R_2,

Asp-d-Ile-Lys-Gly-Lys-Glu-Val-Thr-Val-R_2,

Asp-d-Leu-Lys-Gly-Lys-Glu-Val-Thr-Val-R_2,

Asp-d-Val-Lys-Gly-Lys-Glu-Val-Thr-Val-R_2,

Asp-Cha-Lys-Gly-Lys-Glu-Val-Thr-Val-R_2,

Asp-Ile-d-Lys-Gly-Lys-Glu-Val-Thr-Val-R_2,

Asp-Ile-Arg-Lys-Gly-Lys-Glu-Val-Thr-Val-R_2,

Asp-Ile-homo-Arg-Lys-Gly-Lys-Glu-Val-Thr-Val-R_2,

Asp-Ile-d-Arg-Lys-Gly-Lys-Glu-Val-Thr-Val-R_2,

Asp-Ile-Ile-Lys-Gly-Lys-Glu-Val-Thr-Val-R_2,

Asp-Ile-Met-Lys-Gly-Lys-Glu-Val-Thr-Val-R_2,

Asp-Ile-d-Orn-Lys-Gly-Lys-Glu-Val-Thr-Val-R_2,

d-Gln-Leu-d-Lys-Lys-Gly-Lys-Glu-Val-Thr-Val-R_2,

d-Asp-Leu-d-Lys-Lys-Gly-Lys-Glu-Val-Thr-Val-R_2,

Asp-Ile-Lys-Ala-Lys-Glu-Val-Thr-Val-R_2,

Asp-Ile-Lys-Aib-Lys-Glu-Val-Thr-Val-R_2,

Asp-Ile-Lys-Ala-Lys-Glu-Val-Thr-Val-R_2,

Asp-Ile-Lys-β-Ala-Lys-Glu-Val-Thr-Val-R_2,

Asp-Ile-Lys-Acp-Lys-Glu-Val-Thr-Val-R_2,

d-Asp-Ile-Lys-d-Ala-Lys-Glu-Val-Thr-Val-R_2,

Asp-Ile-Lys-d-Pro-Lys-Glu-Val-Thr-Val-R_2,

Asp-Ile-Lys-Gly-d-Lys-Glu-Val-Thr-Val-R_2,

Asp-Ile-Lys-Gly-Arg-Glu-Val-Thr-Val-R_2,

Asp-Ile-Lys-Gly-l-Arg-Glu-Val-Thr-Val-R_2,

Asp-Ile-Lys-Gly-homo-Arg-Glu-Val-Thr-Val-R_2,

Asp-Ile-Lys-Gly-Met-Glu-Val-Thr-Val-R_2,

Asp-Ile-Lys-Gly-Ile-Glu-Val-Thr-Val-R_2,

Asp-Ile-Lys-Gly-Pro-d-Arg-Glu-Val-Thr-Val-R_2,
Asp-Ile-Lys-Gly-d-Orn-Glu-Val-Thr-Val-R_2,
Asp-Ile-Lys-Gly-Lys-Glu-Val-Met(O)-Val-R_2,
Asp-Ile-Lys-Gly-Lys-Glu-Val-Met-Val-R_2,
Asp-Ile-Lys-Gly-Lys-d-Glu-Val-Thr-Val-R_2,
Asp-Ile-Lys-Gly-Lys-Asp-Val-Thr-Val-R_2,
Asp-Ile-Lys-Gly-Lys-d-Asp-Val-Thr-Val-R_2,
Asp-Ile-Lys-Gly-Lys-Gln-Val-Thr-Val-R_2,
Asp-Ile-Lys-Gly-Lys-Asn-Val-Thr-Val-R_2,
Asp-Ile-Lys-Pro-Lys-d-Glu-Val-Thr-Val-R_2,
Asp-Ile-Lys-Gly-Lys-Glu-Leu-Thr-Val-R_2,
Asp-Ile-Lys-Gly-Lys-Glu-Ile-Thr-Val-R_2,
Asp-Ile-Lys-Gly-Lys-Glu-Met-Thr-Val-R_2,
Asp-Ile-Lys-Gly-Lys-Glu-l-Val-Thr-Val-R_2,
Asp-Ile-Lys-Gly-Lys-Glu-Chg-Thr-Val-R_2,
Asp-Ile-Lys-d-Pro-Lys-Glu-Leu-Thr-Val-R_2,
Asp-Ile-Lys-Gly-Lys-Glu-Val-Ser-Val-R_2,
Asp-Ile-Lys-Gly-Lys-Glu-Val-d-Thr-Val-R_2,
Asp-Ile-Lys-Gly-Lys-Glu-Val-allo-Thr-Val-R_2,
Asp-Ile-Lys-l-Pro-Lys-Glu-Val-Ser-Val-R_2,
Asp-Ile-Lys-l-Pro-Lys-Glu-Val-Leu-Val-R_2,
Asp-Ile-Lys-Gly-Lys-Glu-Val-Met-Val-R_2
Asp-Ile-Lys-Gly-Lys-Glu-Val-Val-R_2
Asp-Ile-Lys-Gly-Lys-Glu-Val-Met-Leu-R_2,
Asp-Ile-Lys-Gly-Lys-Glu-Val-Met-Met-R_2
Asp-Ile-Lys-Gly-Lys-Glu-Val-Met-Chg-R_2,
Asp-Ile-Lys-Gly-Lys-Glu-Val-Met-Valinol,
(Asn-Ile-Lys-Gly-Lys-Gln-Val-Thr-Val)$_2$-NH_2,
(Asp-Ile-Lys-Gly-Lys-Glu-Val-Thr-Val)$_2$-OH,
(Asp-Ile-Lys-Gly-Lys-Glu-Val-Thr-Val)$_2$(Gly)-R_2,
(Asp-Ile-Lys-Gly-Lys-Glu-Val-Thr-Val)$_2$(Pro)-R_2,
(Asp-Ile-Lys-Gly-Lys-Glu-Val-Thr-Val)$_2$β-Ala-R_2,
Cyclo(-Asp-Ile-Lys-Gly-Lys-Glu-Val-Thr-Val-),
Cyclo(-Asp-Ile-Lys-Gly-Lys-Gln-Val-Thr-Val-Pro),
Cyclo(-Asp-Ile-Lys-Gly-Lys-Glu-Val-Thr-Val-Gly),
Cyclo(-Asp-Ile-Lys-Gly-Lys-Glu-Val-Thr-Val-β-Ala), and the

polymeric peptides $(Asp\text{-}Ile\text{-}Lys\text{-}Gly\text{-}Lys\text{-}Glu\text{-}Val\text{-}Thr\text{-}Val)_n\text{-}R_2$, wherein n is an integer from 3 to 20, and

wherein R_2 is selected from the group consisting of OH, NH_2, OR_3, OR_3OH, and NHR_3, with R_3 being selected from 1 to 4 carbon atom alkyls and 6 to 10 carbon atom aryls.

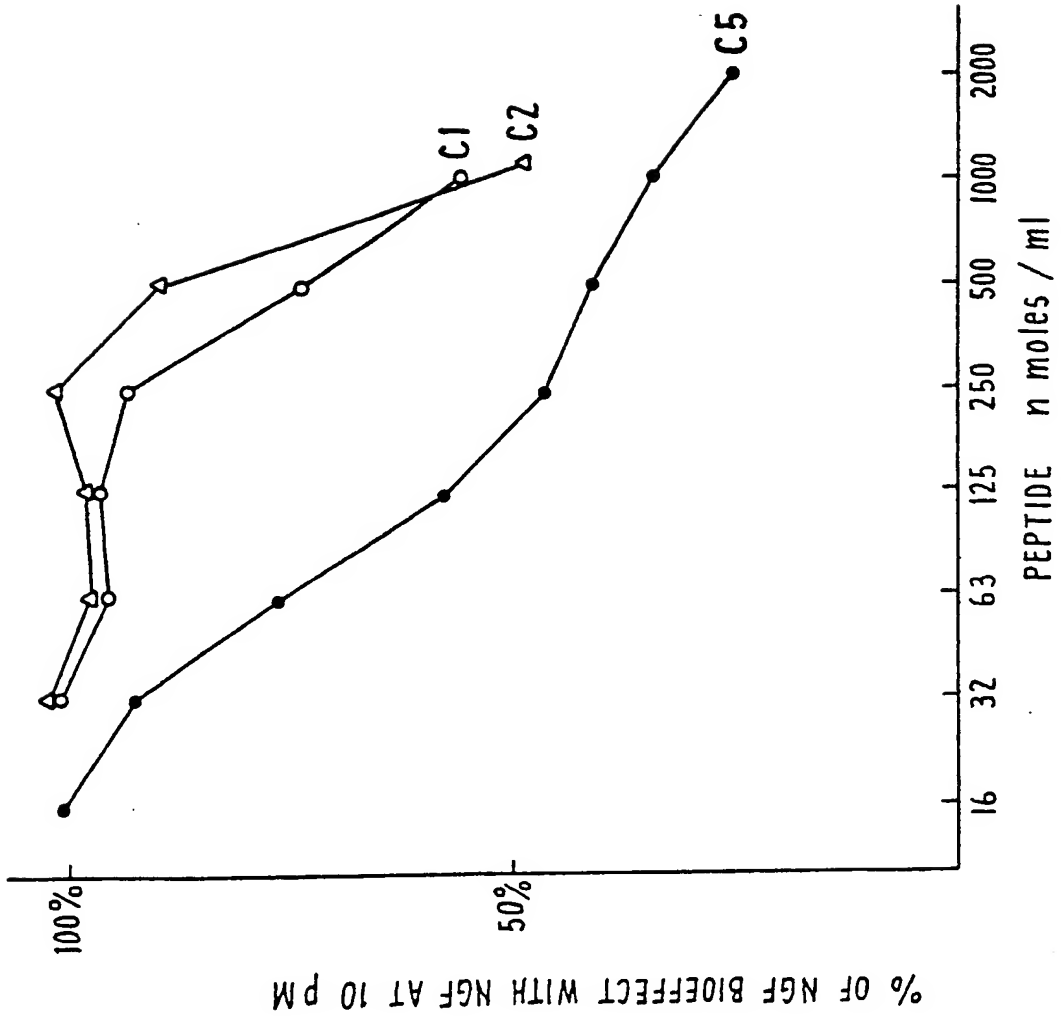
12. A method of inhibiting NGF-mediated biochemical or biophysical cellular effects which comprises applying an NGF antagonist peptide of claims 1-11 to NGF-responsive cells.

13. A method for treatment of disorders involving NGF-responsive cells, including degenerative neurologic disorders of the central nervous system, especially Alzheimer's disease, Down's syndrome, disorders associated with β-protein, or results from normal aging and neoplastic disorders which method comprises:

administering an effective amount of a NGF peptide of claims 1-11 to an individual suffering from such a disorder.

14. A pharmaceutical composition comprising an effective amount of the peptide of claims 1-11 and a pharmaceutically acceptable excipient.

FIG. 1A.


C5
C1
C2
16
32
63
125
250
500
1000
2000
PEPTIDE n moles / ml
100%
50%
% OF NGF BIOEFFECT WITH NGF AT 10 pM

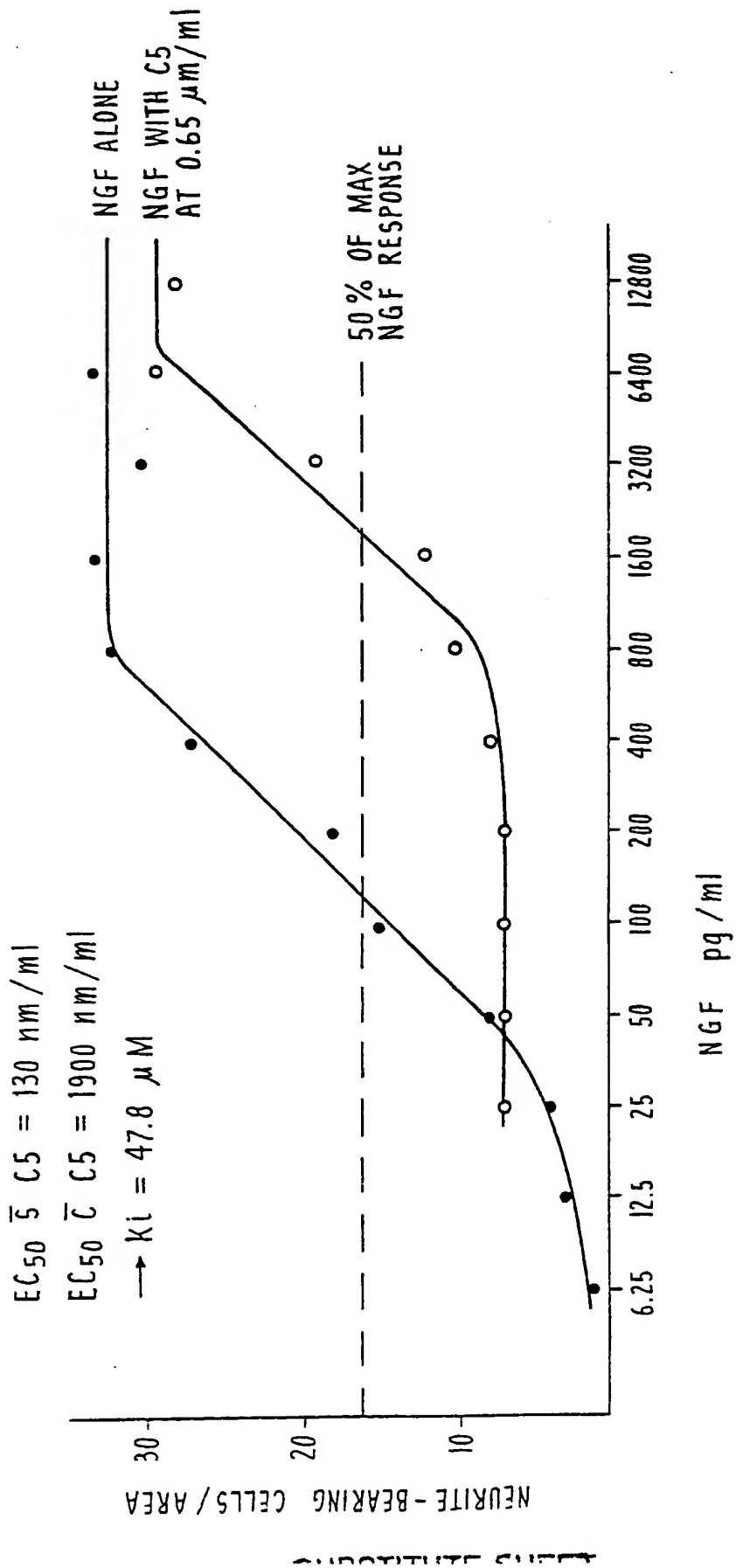

NGF ALONE
NGF WITH C5
AT 0.65 μm/ml
50% OF MAX
NGF RESPONSE
EC50 S̄ C5 = 130 nm/ml
EC50 C̄ C5 = 1900 nm/ml
→ Ki = 47.8 μM
NEURITE-BEARING CELLS / AREA
30
20
10
6.25
12.5
25
50
100
200
400
800
1600
3200
6400
12800
NGF pg/ml

FIG. 1B.

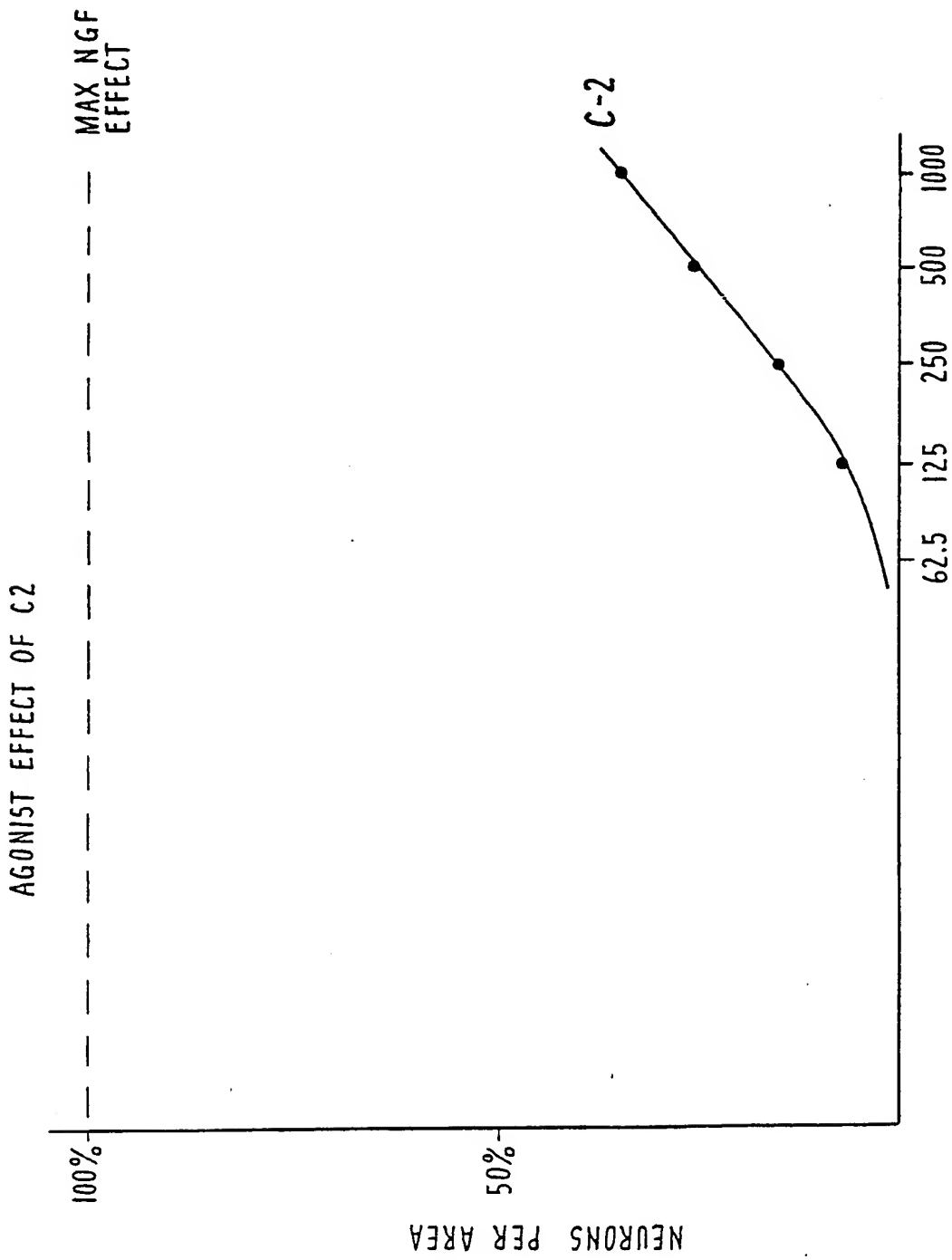

MAX NGF EFFECT
AGONIST EFFECT OF C2
C-2
100%
50%
NEURONS PER AREA
62.5
125
250
500
1000
n moles / ml OF PEPTIDE

FIG. 2.

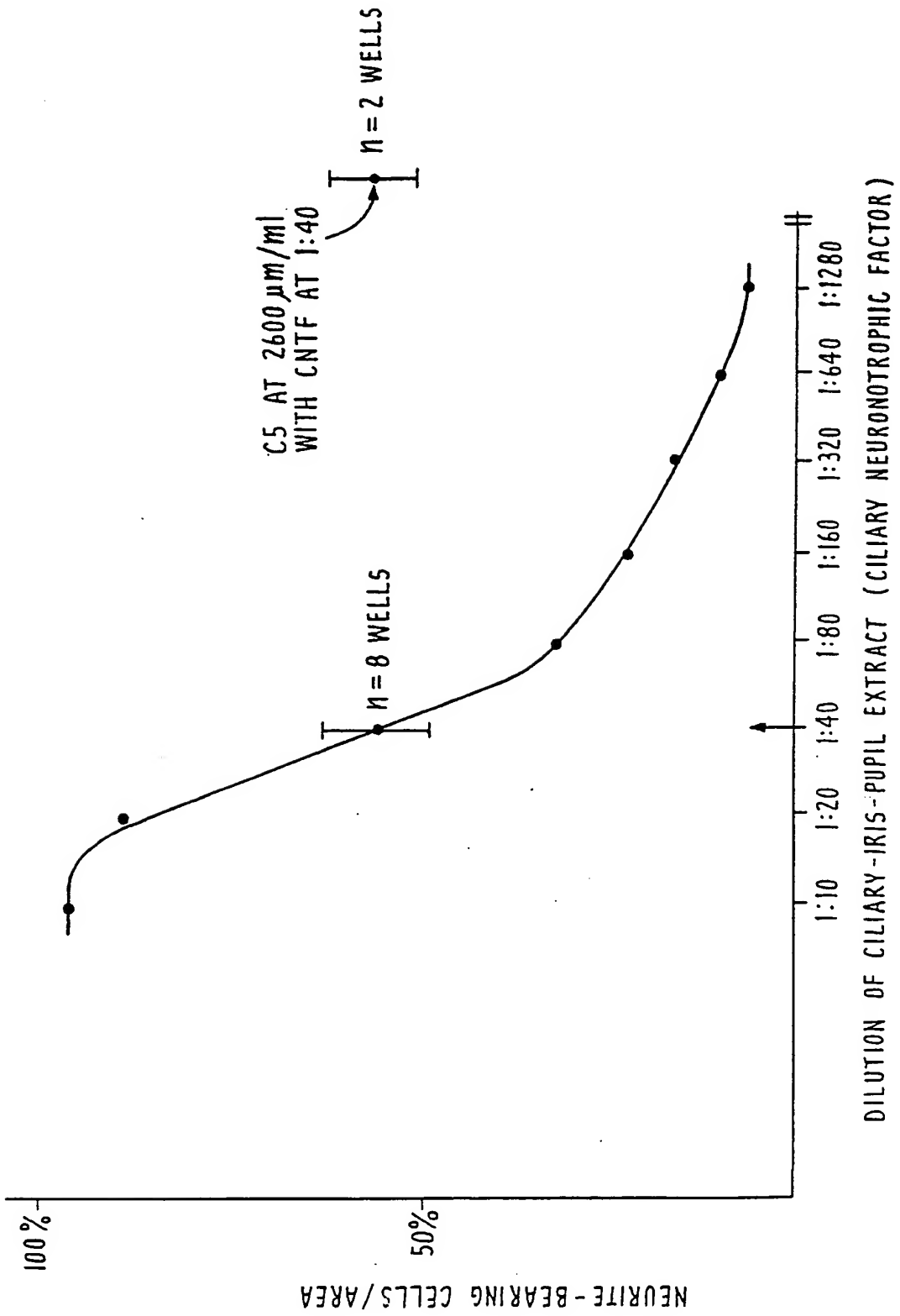

n = 2 WELLS
C5 AT 2600 µm/ml
WITH CNTF AT 1:40
n = 8 WELLS
100 %
50%
NEURITE-BEARING CELLS/AREA
1:10
1:20
1:40
1:80
1:160
1:320
1:640
1:1280
DILUTION OF CILIARY-IRIS-PUPIL EXTRACT (CILIARY NEUROTROPHIC FACTOR)

FIG. 3.

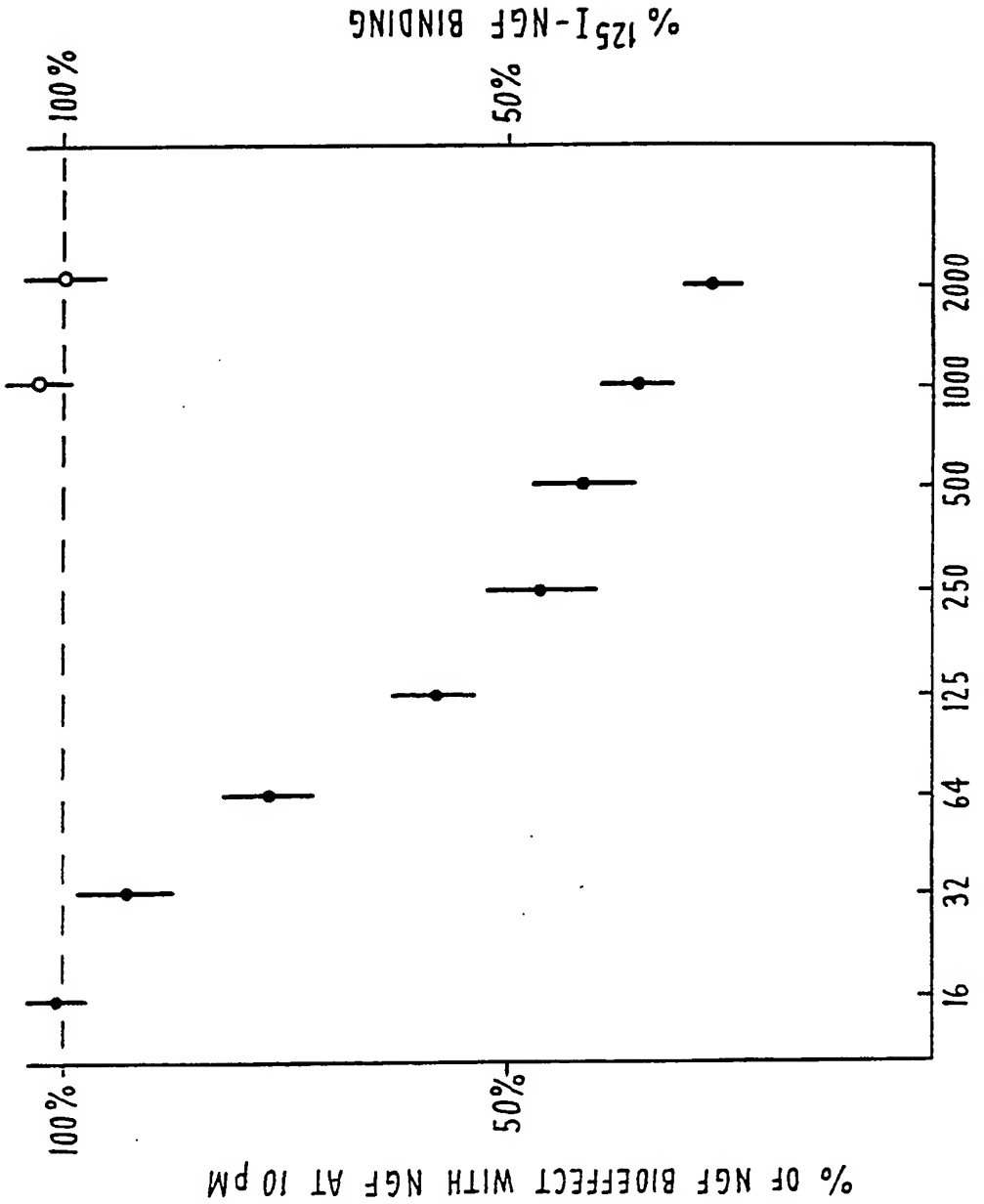

% ^{125}I-NGF BINDING
100%
50%
2000
1000
500
250
125
64
32
16
C5 n moles / ml
100%
50%
% OF NGF BIOEFFECT WITH NGF AT 10 pM

FIG. 4.

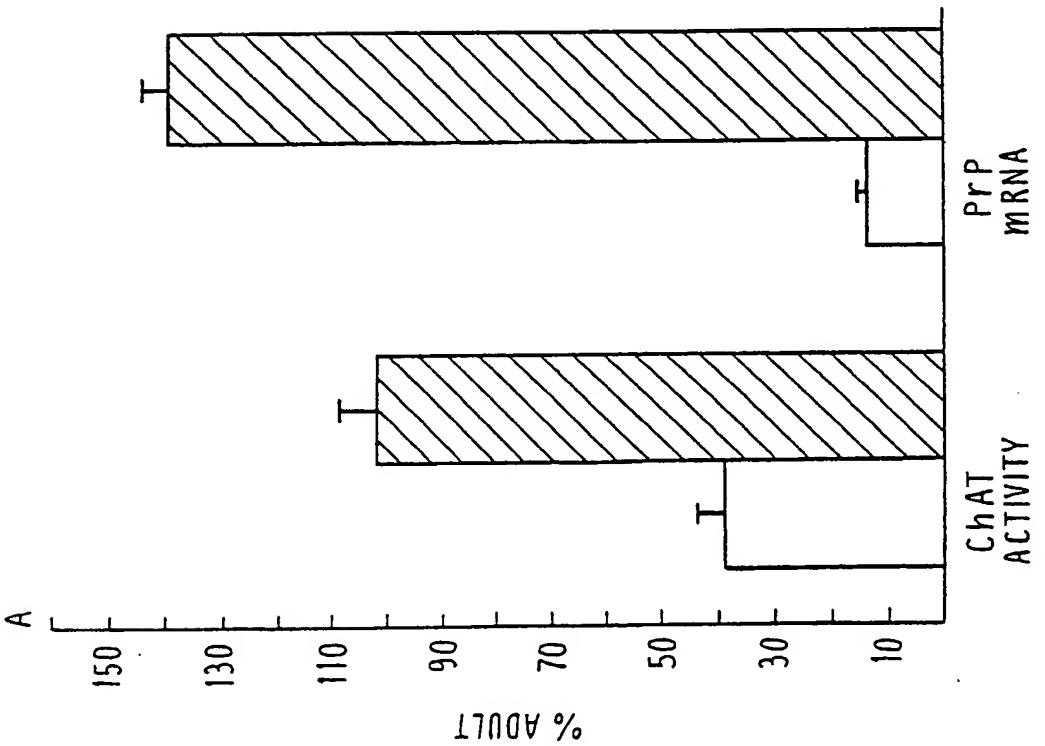


FIG. 5A

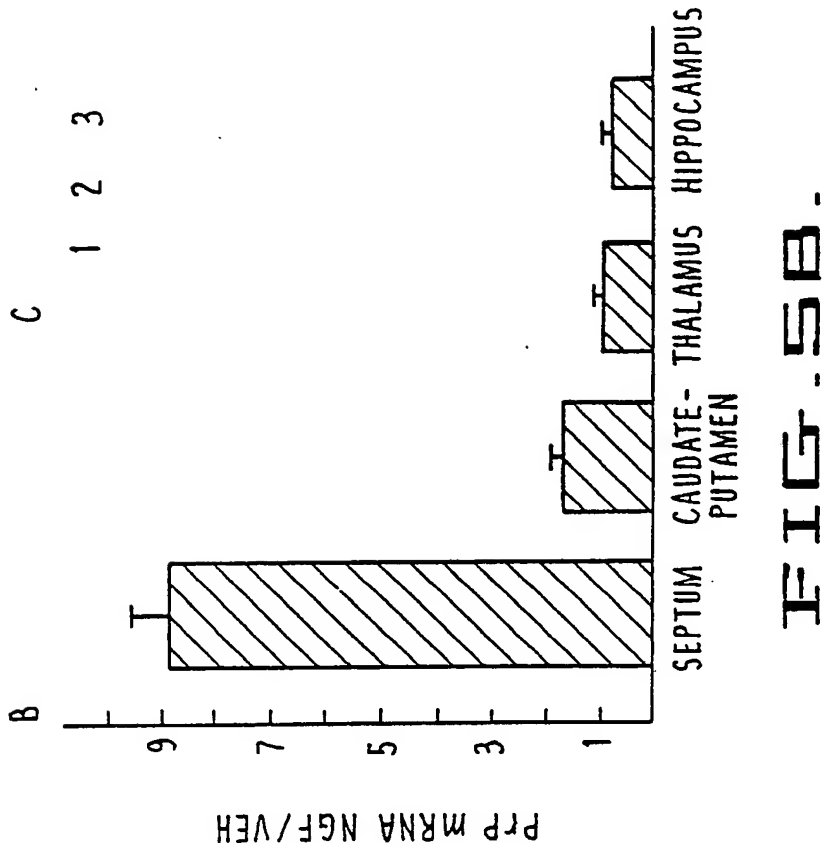


FIG. 5B.

SUBSTITUTE SHEET

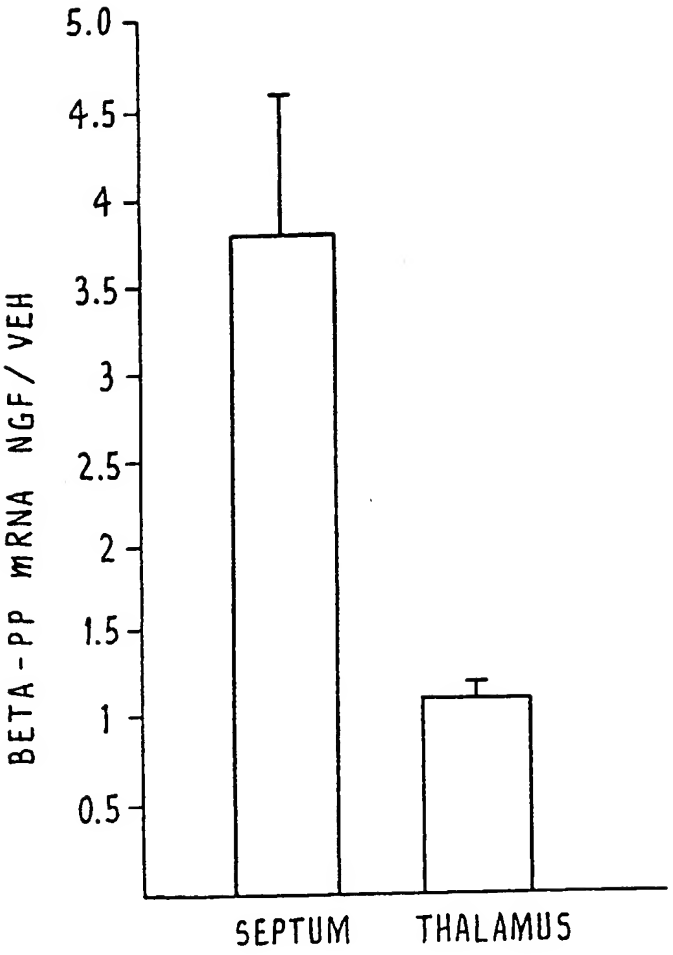


FIG. 6.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US89/01128

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(4); C 07 K 5/00

II FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.	530/330; 514/18

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

Chemical Abstracts On-Line,
Automated Patent System

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
P,A	U.S., A, 4,746,508, Carey, Published 24 May 1988.	1,2-6, & 11-13
A	Puma, Chemical Abstracts, 98 (17): 137747 s, Published 1983.	1, 2-6, & 11-13
A	Montalcini, Science, 237, 1154-1162, Published 4 September 1987.	1, 2-6, & 11-13
A	Hefti, Annals of Neurology, 20, 275-281, Published 3 September 1986.	1, 2-6, & 11-13
A	Carrell, Nature, 331, 478-479 Published 1988.	1, 2-6, & 11-13
A	Tanzi, Science, 235, 880-884 Published 1987.	1, 2-6, & 11-13
A	Korsching, TINS, 570-573 Published 1986.	1, 2-6, & 11-13

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
27 June 1989	26 JUL 1989
International Searching Authority	**Signature of Authorized Officer**
ISA/US	Susan M. Learned

Form PCT/ISA/210 (second sheet) (Rev.11-87)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	Meier, EMBO Journal, 5(7), 1489-1493, Published 1986.	1
Y	Romani, International Journal of Peptide Protein Research, 29, 107-117, Published 1987	1
A	Mercanti, Biochimica et Biophysica Acta, 496, 412-419, Published 1977.	1, 2-6, & 11-13
A	U.S., A, 4,230,691, Young, Published 28 October 1980.	1, 2-6, & 11-13

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers . because they relate to subject matter [12] not required to be searched by this Authority, namely:

2. ☐ Claim numbers .... , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

3. ☐ Claim numbers_____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

See attachment.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☒ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

Claims 1, 2-6, and 11, with an election of species C5, and claims 12 and 13. (Telephone practice.)

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Attachment to Form PCT/ISA/210, VI. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING

This application contains claims directed to the following patentably distinct species of the claimed invention:

Peptides, of varying lengths, defined by residues
1) 20-48 (claims 1, 2-6, and 11)
2) 50-84 (claims 1, 7-8, and 11)
3) 98-106 (claims 1, 9-10, and 11)
of Nerve Growth Factor.

Each of these peptides, although they may have the same utility, are patentably distinct species. Having the same functionality does not make them obvious variants of one another. Each of these peptides are fragments derived from different regions of Nerve Growth Factor, each of the peptides have unique amino acid compositions, and each of the peptides vary in length. As well, peptides that are three residues long do not make obvious peptides that are ten or twenty-eight residues long.

Applicant is entitled to a product, a method of use, and a method of preparing said product, under PCT Rule 13.2. Applicant has claimed the following peptide composition and two distinct methods:

I. Claims 1-11, and 14, drawn to an NGF peptide, and a pharmaceutical compositions using said peptide, classified in Class 530, subclass 324-331.

II. Claim 12, drawn to a method of inhibiting NGF-mediated biochemical or biophysical cellular effects, classified in Class 514, subclass 12-18.

III. Claim 13, drawn to a method of treatment by administration of NGF peptides, classified in Class 514, subclass 12-18.